Journal of Embryology and Experimental Morphology

VOLUME 1 *June 1953* PART 2

PUBLISHED FOR THE COMPANY OF BIOLOGISTS LIMITED

OXFORD : AT THE CLARENDON PRESS

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GEOFFREY CUMBERLEGE
OXFORD UNIVERSITY PRESS, AMEN HOUSE, LONDON, E.C.4

Bone-marrow of the Guinea-pig as a Mesodermal Inductor in Implantation Experiments with Embryos of *Triturus*

by SULO TOIVONEN[1]

From the Zoological Laboratory of the University of Helsinki

WITH ONE PLATE

INTRODUCTION

LEVANDER (1938) has shown that an alcohol extract prepared from bone can, when injected into rabbit muscle, induce there cartilage and bone cells. This observation has been confirmed by several investigators (Annersten, 1940; Bertelsen, 1944; Lacroix, 1945; Levander, 1949; Willestaedt, Levander & Hult, 1950). Of these, Bertelsen has shown in addition that not only is an extract prepared from bone proper active, but also that an extract prepared from bone-marrow is even more active. In addition to the authors mentioned above, Schreiber (1950) has lately dealt with the problem. He has assumed, on good grounds, that the agent inducing bone and cartilaginous tissue in the experiments mentioned above is similar, as regards its chemical nature, to the 'spinal' inducing agent in my experiments (Toivonen, 1940, 1949 *a* and *b*, 1950). This assumption of Schreiber's has prompted me to investigate experimentally the inducing action of bone-marrow when implanted into the gastrula of *Triturus*.

MATERIAL AND METHODS

Gastrulae of *Triturus vulgaris* were used as the host in all the experiments. The inductor material was marrow from the thigh-bone of the guinea-pig, which had been immersed in 70 per cent. alcohol for approximately 24 hours. Operations were carried out by the implantation method, using Holtfreter saline with sodium sulphadiazine added as a bactericidal agent. Embryos were cultured usually for 2 weeks. Specimens were fixed in Bouin's fluid and first stained in bulk with borax-carmine; the sections were stained further with Picroblau-schwarz.

RESULTS

On external examination of the experimental animals the structures induced by the bone-marrow appeared very similar to those caused by alcohol-treated

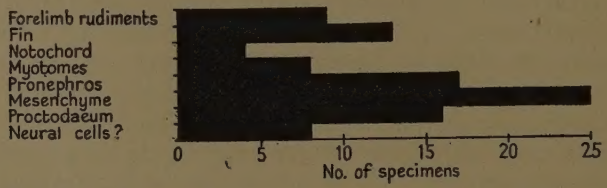
[1] *Author's address*: Zoological Laboratory, University of Helsinki, P.-Rautatiek. 13, Helsinki, Finland.

[J. Embryol. exp. Morph. Vol. I, Part 2, pp. 97–104, June 1953]

kidney tissue in my earlier experiments (1940, 1949 *a* and *b*, 1950). In those of the experimental animals in which induced structures could be seen externally, tail-like appendages were common (40 per cent.), and an irregular fin was found in many others (24 per cent.). The inductor thus seemed to have a 'spinal' inducing action.

Altogether induction occurred in thirty animals. Microscopic preparations showed that in five animals the extra formation was in continuity with the central nervous system of the host. In all these cases a very complete extra tail was found, containing, in addition to the notochord and rows of paired myotomes, a spinal cord. Since in these specimens the connexion between the induced structure and the host was so direct, they will not be discussed here (cf. Holtfreter, 1936, p. 532; Toivonen, 1940, p. 19).

An analysis was made of the frequency of the various types of differentiated structures induced in the remaining twenty-five animals. The results are seen in Text-fig. 1.



TEXT-FIG. 1. Diagram showing the frequency of the various differentiations induced in the twenty-five embryos analysed.

A scrutiny of the preparations reveals the surprising fact that no actual neural tissue was ever induced. Not even unspecialized neural vesicles are found in the induced structures. In some of those animals (32 per cent.) in which a tail-like formation was externally visible the mesenchyme, containing numerous melanophores, also contained isolated, more darkly staining cells, which might perhaps be regarded as neural cells. It has not, however, been possible to confirm the neural nature of these cells, and the presence of neural cells is therefore still to be regarded as questionable.

The weakest level of mesodermal induction is represented by increased mesenchyme in the host at the site of the implant. This mesenchyme usually contained numerous melanophores. In nine cases (36 per cent.) one or two extra forelimb rudiments had been produced (Plate, figs. B and C). In many cases large blood lacunae (Plate, fig. A) were found in connexion with the induced structures. These have not been taken into account in the analysis, since there is no means of discovering whether they were induced or were caused mechanically by the implant. Myotomes (Plate, figs. A and B) and notochord (Plate, fig. C) occur relatively seldom in the inductions (32 and 16 per cent.). On the other hand, a typical pronephros (Plate, fig. C) with its Wolffian ducts was common (68

per cent.). If an extra proctodaeum with its anal opening was found in the animal, the paired induced Wolffian ducts usually opened into it. An extra anal opening and proctodaeum (Plate, fig. D) occurred in 64 per cent. of the twenty-five experimental animals included in the analysis. The proctodaeum was in most cases connected with the intestine of the host, although the connexion was usually extremely narrow and in my opinion secondary. In three cases anus and proctodaeum were quite independent of the host intestine. It is worth noting that the extra anal opening can occur even at the level of the anterior portion of the liver; in most cases, however, it was found in the hind part of the trunk.

DISCUSSION

The inducing action of alcohol-treated bone-marrow is to be regarded as solely mesodermal, except for some possibly neural cells. For the ectodermal proctodaea were, in my opinion, secondarily evoked by the induced mesoderm (cf. Holtfreter, 1936, p. 521), and not directly caused by the inductor. Alcohol-treated bone-marrow is thus a very specific mesoderm-inductor. In this respect it is more specific in its action than any killed inductor previously used. For instance, the alcohol-treated kidney tissue of the guinea-pig, used in my earlier experiments (Toivonen, 1940, 1949 *a* and *b*, 1950), is less specific in its action, since in addition to mesoderm it also regularly induces spinal cord and parts of deuterencephalon together with ear vesicles. Extraction of this kidney tissue in petroleum ether changes its action so that the neural component in the induction is weakened, parts of the brain as a rule do not appear, and the spinal cord is thinner; and in certain individual cases no more than an isolated myotome has occurred, without any neural structures. Extraction in petroleum ether thus changes the inducing action of kidney tissue of the guinea-pig in the direction of mesoderm-induction alone; and it can be assumed that if the extraction could be done very thoroughly, the induction might be similar to that obtained in this investigation with alcohol-treated bone-marrow. The experiments in the present investigation in my opinion show unequivocally that the mesoderm-inducing agent is entirely independent of the agents inducing neural structures. The spinal agent earlier postulated can thus be further divided into components inducing mesoderm and inducing neural structures.

The question now naturally arises as to what is the specific action, independent of the mesoderm-inducing agent, of the neural inductor component contained in the 'spinal' inductor. My earlier experiments with kidney tissue of the guinea-pig, which showed a weak power of archencephalic induction after heat treatment, would seem to indicate that it is archencephalic. We would then be faced with a system of two opposing gradients of agents, which Lehmann (1950, p. 144) has suggested as a possibility: much archencephalic + little mesodermal = archencephalic action; moderate archencephalic + moderate mesodermal = deuterencephalic action; little archencephalic + much mesodermal = spinal action. Kuusi (1951, p. 87), too, regards such a system as probable. I have since,

however, shown (Toivonen, 1951, 1952), when comparing the structures induced by liver tissue of starved and of well-fed guinea-pigs, that during starvation the action of the liver changes in an archencephalic direction; the deuterencephalic component contained in the liver of a well-fed guinea-pig then disappears almost entirely. As an explanation for this phenomenon I regarded it as probable that during starvation a deuterencephalic agent of large molecular size splits into an archencephalic one of small molecular size. Both would thus be rather closely related chemically, but in fact different substances. Since the same number of spinal structures was induced by the liver of both starved and well-fed guinea-pigs, it is evident that the change which occurs does not affect the spinal agent contained in the liver.

It is noteworthy, however, that in no case has liver tissue of either well-fed or starved guinea-pigs induced a typical tail containing a spinal cord. In my opinion the interpretation of this fact must be that the inductor has not contained enough of the mesoderm-inducing agent necessary for this differentiation. For it is possible that the production of the spinal cord is actually brought about by the autonomous growth of the mesoderm, especially the myotomes, which, whilst the tail-bud is developing, at the same time stretches the induced neural structure into the spinal cord.

The present experiments with bone-marrow in any case exclude the possibility that the spinal cord and the neural component in the extra tails can have been secondarily induced by the mesoderm. The ectoderm is apparently by this time already so 'aged' that its competence no longer allows the production of an induced structure of this kind. The prerequisite for the production of a complete tail is thus that the inductor itself should contain adequate amounts of both the mesodermal and the neural agent.

My earlier assumption mentioned above that the neural-inducing agent would prove further divisible into two different components, archencephalic and deuterencephalic, was then advanced as a working hypothesis, and it has not yet been possible to confirm it. If it is true, the deuterencephalic agent contained in the inductor would in my opinion be primarily responsible for the spinal cord produced in connexion with tail formation. Such an assumption is justified by the regional sequence of these structures in the normal embryo.

As regards the opinion that deuterencephalic induction also requires the mesoderm-inducing agent for its occurrence (cf. p. 99), I do not regard it as necessary. Actual direct evidence for this opinion of mine I am not able to present. Indirectly it is supported by experimental series in which no ordinary mesodermal structures occur among the archencephalic and deuterencephalic structures induced. In my earlier material I have discovered isolated cases of this kind. Of the inductors used by Kuusi (1951), the following, for instance, have had such an action: different nuclear fractions of the liver and kidney of the guinea-pig (p. 24, Fig. 9), certain nucleoprotein and plasma protein fractions (p. 33, Fig. 30), and the large granule fractions prepared from the liver of the

same animal (p. 47, Fig. 54). If these inductors were to contain the mesoderm-inducing agent taking part in the induction of deuterencephalic structures, then this agent ought in some specimens to manifest its own effect and at least produce a weak mesodermal reaction. Since this has not happened, the participation of the mesoderm-inducing agent in the induction of deuterencephalic structures is in my opinion very questionable.

This view is more directly supported by the experimental series of Chuang (1939, 1940), in which he investigated the effect of boiling of different duration on the nature of the structures induced by kidney tissue of the mouse and by liver tissue of the newt. In the structures induced by the former no mesodermal material occurred if boiling had lasted over 5 minutes; from the latter, the ability to induce mesoderm had already disappeared in 2 seconds. On the other hand, even after boiling for 1 hour mouse kidney tissue induced parts of the brain and even ear vesicles, and with liver tissue of the newt the frequency of induced ear vesicles and parts of the brain even increased after the ability to induce mesoderm had been lost. Chuang does not analyse the nature of the parts of the brain induced, but since ear vesicles can be regarded as indicators of the deuterencephalic region, their occurrence is evidence of deuterencephalic differentiation in the inductions. But it cannot be thought that the thermolabile mesoderm-inducing agent could survive such a prolonged boiling treatment. Is it not more probable that deuterencephalic induction had occurred without any mesoderm-inducing agent in the inductor?

It was Chuang who first showed conclusively the thermolability of the mesoderm-inducing agent. Later this has been confirmed at least by me and by Okada (1948) and Rotman (Toivonen, 1950, p. 52). Okada's experimental results are interesting because the inductor used by him, the skin of the abdomen of *Rana pyrrhogaster*, when explanted fresh to an ectodermal vesicle of *Triturus* induced a purely mesodermal structure similar to that induced by the alcohol-treated bone-marrow used in the present investigation. When Okada further treated pieces of skin for some minutes in boiling water before implantation, only neural and epidermal structures were induced; these structures were, in my opinion, mainly archencephalic. Okada interprets his experimental result by adopting the possibility suggested by Chuang (1939) that the mesoderm-inducing agent changes during heat treatment into a neural-inducing agent. In my opinion this interpretation is hardly likely to be correct. In this case the mesoderm-inducing agent would seem to be destroyed and the neural agent at the same time liberated. It can be regarded as probable that from a fresh piece of skin only mesoderm-inducing agent can be liberated because it occurs in the corium of the ventral skin. After heat treatment those agents can also be liberated which until then have been situated within the poorly permeable epidermal cells, and after the destruction of the mesoderm-inducing agent these may cause a neural induction. It would be interesting to study what would be the effect of treating the fresh skin with alcohol before implantation. The mesoderm-inducing agent

would not then be destroyed, but the original restriction of the release of the substances caused by the poor permeability of the living cells would be eliminated. I would expect that both mesodermal and neural elements would then be induced. Thus in my opinion the mesoderm-inducing agent does not change into the neural one.

Waddington (1952) has recently referred to the experiments of Barth & Graff (1943), and other corresponding experiments, in which the inductor has in one way or another been killed and then explanted into ectodermal vesicles. In these experiments the regionally specific action of the inductor has for the most part been eliminated and above all no mesoderm was induced. Referring to my earlier schematic picture (Toivonen, 1940, p. 135, Fig. 45) I must emphasize that the natural inductor consists of embryonic tissue, in which no great regional differences in the concentrations of substances in the cells can prevail, since no differentiation has yet taken place. When alive, the different regions of the archenteron roof are specific in their action in my opinion mainly because the permeability of the cells is different in different regions, and this regulates the passage of active substances in a way characteristic of each region. When the tissue is killed in one way or another, all the substances contained in it are liberated to compete for the reactive material. Those of the active substances which are overwhelming in amount, or are first able to determine the reactive material, determine the nature of the structures induced. It is evident that the mesoderm-inducing agent in a killed natural inductor is always defeated in this competition when used in explantation experiments. In Waddington's (1952) latest explantation experiments, in which he has used rather old donors and in addition destroyed the mesoderm-inducing agent with hot water, it appears that the differentiation of the concentration of the neural agents in the different regions has already started. The induction of brain or eye was not uncommon when anterior inductors were used, but was not seen at all with posterior ones. In highly differentiated tissues of adult animals, such as bone-marrow, liver, and kidney, this chemical differentiation has advanced so far that the tissues, even if killed in alcohol, are more or less specific in their action when used as heterogenous inductors. In my opinion, therefore, the inability of the alcohol-killed natural inductor to induce any but neural structures in explantation experiments is only due to the fact that the neural agents camouflage the mesoderm-inducing agent contained in the inductor. I assume that the mesoderm-inducing agent would exert its effect if the neural agents of the inductor were diminished, for instance by extraction with petroleum ether.

SUMMARY

1. Marrow from the thigh-bone of the guinea-pig, treated in 70 per cent. alcohol, has been used as the inductor material in implantation experiments with gastrulae of *Triturus*.

2. The inductor has induced mesodermal structures such as limb rudiments,

notochords, pronephric tubules, and mesenchyme, and, in addition to these, proctodaea with anal openings. These last are regarded as secondary induction products of the induced mesoderm. The only suggestion of neural induction consisted of darkly staining cells, which may perhaps be regarded as neural.

3. The results show that the mesoderm-inducing agent is independent of the agents inducing neural structures, and the 'spinal' agent earlier postulated can thus be further divided into components inducing mesoderm and inducing neural structures.

4. The question as to what is the specific action, independent of the mesoderm-inducing agent, of the neural-inducing component in the previously established 'spinal' inductor, is discussed.

Financial support for this work has been received from the Niilo Helander Foundation, for which I wish to express my gratitude.

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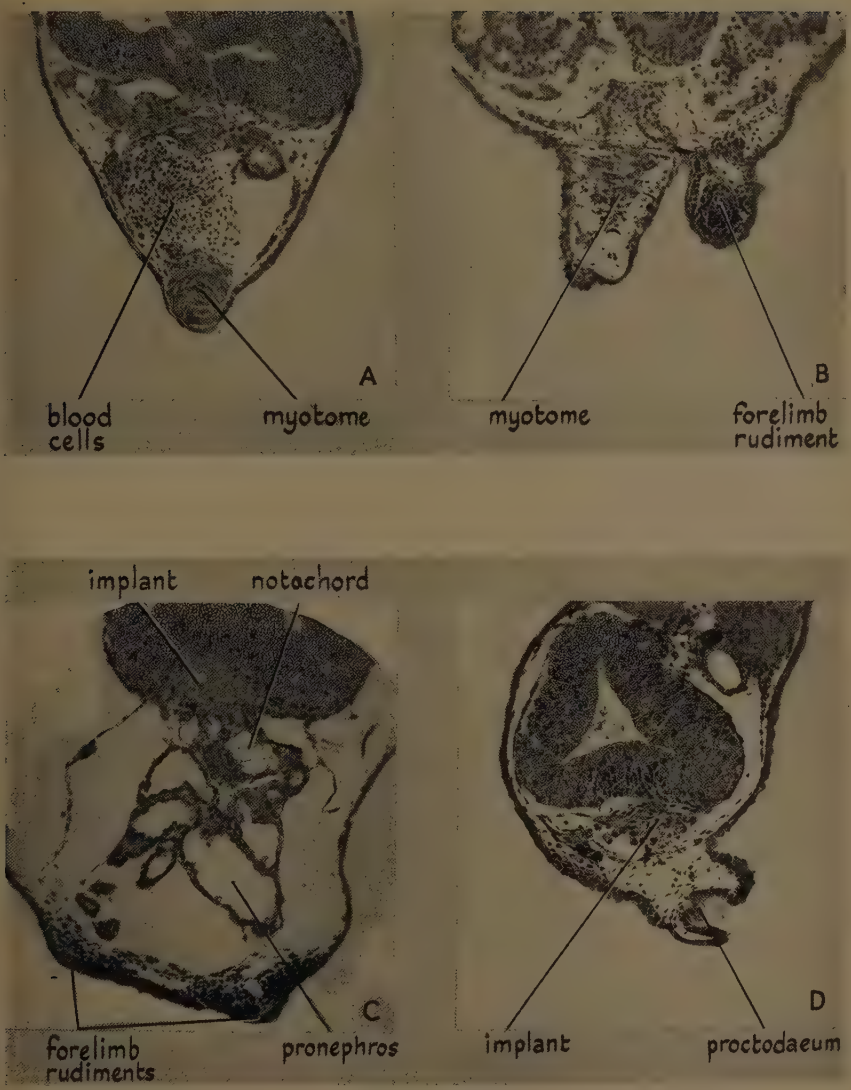
EXPLANATION OF PLATE

FIG. A. Section through an embryo with myotomes and blood-cells induced ventrally.

FIG. B. Section through the liver region of an embryo with an induced forelimb rudiment and myotomes.

FIG. C. Section through an embryo in which two forelimb rudiments, a notochord, and pronephric tubules have been induced.

FIG. D. Section through an embryo with an induced ectodermal proctodaeum.



S. TOIVONEN

Plate 1

Metabolism and Glycogen Formation in the Liver of the Chicken Embryo

by R. J. O'CONNOR[1]

From the John Burford Carlill Pathological Laboratories, Westminster School of Medicine

INTRODUCTION

THE investigations of Dalton (1937) and of Lee (1951) have shown that glycogen appears in the liver of the chicken embryo at 7–8 days' incubation and before the formation of insulin by the islets of Langerhans at 11–12 days (Potvin & Aron, 1927). Further, Dalton (1937) has shown that when livers of embryos of 3 days' incubation were transplanted on to the chorio-allantoic membrane of 11–13-day hosts there was no acceleration of glycogen formation, althought it can be presumed that the transplanted livers were under the influence of insulin and other hormones in the blood-stream of the host. It is possible, therefore, that the first formation of glycogen is relatively independent of factors reaching the liver by way of the blood-stream and to be related more to events originating within the liver cells themselves. Such a possibility is in accordance with the results obtained in cultures of embryonic liver by Nordmann (1929), who showed that the formation of glycogen was, to a large extent, independent of the composition of the culture medium.

If the initial deposition of glycogen is related to changes in the liver cells it is possible that the glycogen deposition is associated with loss of the ability to break down carbohydrate, storage of carbohydrate replacing its utilization. This possibility has been investigated by comparing, at different stages of development, the glycogen content and the carbohydrate metabolism of the developing liver in the chick embryo.

MATERIAL AND METHODS

The eggs used were of no particular strain and were incubated at 38° C. There was considerable variation in the stage of development reached at any given time of incubation. Therefore, to facilitate comparison of the observations made, the embryos were grouped into arbitrarily chosen stages of development. Each of these stages represents an increase in the eye diameter of 0·7 mm., and each stage is referred to by its median diameter. Thus results are recorded for the develop-

[1] *Author's address*: Westminster School of Medicine, Horseferry Road, London, S.W.1, England.

[J. Embryol. exp. Morph. Vol. I, Part 2, pp. 105–14, June 1953]

mental stages of eye diameter 2·5 mm., 3·2 mm., and so on. The mean time of incubation for each of these stages was determined and is recorded in Table 1. The various observations to be described were not made in any particular order and were distributed at random among the many batches of eggs used in making the observations.

TABLE 1

Liver of chicken embryo. Mean values and standard error of the mean at different stages of development for time of incubation, and dry weight expressed as the percentage of wet weight

Stage of development, eye diameter (mm.)		*Time of incubation (days)*			*Dry weight % of wet weight*		
Range	*Median*	*N.*	*M.*	*S.E.*	*N.*	*M.*	*S.E.*
2·2–2·8	2·5	18	5·5	0·15	5	21	1·3
2·9–3·5	3·2	12	6·1	0·12	5	19	0·8
3·6–4·2	3·9	9	6·5	0·14	4	21	0·5
4·3–4·9	4·6	10	6·9	0·14	5	22	2·4
5·0–5·6	5·3	14	7·3	0·11	5	21	0·9
5·7–6·3	6·0	17	7·8	0·06	4	23	1·0
6·4–7·0	6·7	20	8·4	0·21	4	24	1·2
—	—	—	10th day	—	4	24	1·2

N. = Number of observations.
M. = Mean.
S.E. = Standard error of mean.

The estimation of glycogen

Dalton (1937) and Lee (1951) have previously recorded the variations in glycogen content of the liver at different days of incubation, but it was considered desirable to repeat these observations on embryos incubated under the same conditions as those used to measure liver metabolism. Glycogen content was therefore estimated by the histochemical technique of Pritchard (1949), which is based on the reduction of silver diamino hydroxide by the glycogen after preliminary treatment with chromic acid. When sections reacted positively, confirmatory evidence of the presence of glycogen was obtained by the failure of the reaction to appear in corresponding sections treated with salivary diastase. In some cases sections were stained with Best's carmine technique as described by Carleton & Leach (1938); the results corresponded to those obtained by the silver reduction method. In all cases, in order to minimize loss of glycogen, the embryos were placed in the fixative immediately after removal from the shell, and removal of the liver for sectioning was carried out in this fluid (ethyl alcohol 90 per cent., formalin 10 per cent.).

Since the main purpose of the examination was to establish the time at which glycogen first appeared under the conditions of the observations, it was not necessary to measure it quantitatively. However, a subjective comparison of the con-

centration of glycogen at various stages of development was made by scoring each liver as 0–3 arbitrary units and thus making a comparative assessment of the glycogen concentration for each of the stages of development.

The estimation of the rate of respiration and the respiratory quotient

In order to estimate the carbohydrate metabolism of the liver these measurements were made on isolated livers by means of a modified Cartesian diver micromanometer (O'Connor, 1948, 1950). Embryos were removed from the shell and placed in the following medium: NaCl 0·9 g., KCl 0·02 g., $MgCl_2$ 0·02 g., $CaCl_2$ 0·02 g., water 100 ml., to which was added 10 ml. M/15 phosphate buffer (Sørensen) to produce pH 7·4. In one series of observations the medium contained glucose 0·20 g./100 ml., while in another series the glucose was omitted from the medium. All subsequent manipulations and the actual measurements were made in these media.

Livers were dissected from the embryos and portions removed from the ventral aspect of the right lobe with iridectomy scissors. These portions were trimmed to a thickness of 0·2–0·3 mm. and to a volume 0·5–0·8 c.mm. The volume was measured by compressing the tissue beneath the coverslip of a cell-counting chamber, the depth of which was 0·1 mm. (O'Connor, 1950). The amount of compression was adequate to express any blood remaining in the vascular spaces and to obliterate the spaces. The volume of the tissue was calculated from the area covered when the tissue was compressed in this way. After determination of its volume each portion of liver was introduced into a Cartesian diver to which fluids were added according to the arrangement, described previously, for the measurement of the rate of respiration and the respiratory quotient (O'Connor, 1950). The total capacity of the divers used in the present observations was 50–60 μl. and the portions of liver were suspended in 15–20 μl. medium. Control divers were used to estimate the amount of carbon dioxide in the liver fragments and in the diver fluids at the beginning of the observations. The rate of respiration and the respiratory quotient were estimated for a period of 2–3 hours, during which time there was no significant variation in the former.

Experiments were also performed to find out whether the rate of respiration and the respiratory quotient were affected by the compression applied to the liver portions when the volume was determined. The respiratory quotient was determined without measuring the volume of the tissue, and the rate of respiration when the volume was measured after, instead of before, the manometric measurements. Results obtained in this way did not differ significantly from those obtained when corresponding liver portions were investigated in the manner described above.

Measurement of dry weight and wet weight of the liver

Since rates of respiration are related to unit volume of liver substance, it is possible that the rates so expressed might be modified by the degree of hydration

of the liver cells. Therefore, at the various stages of development, the dry weight of the liver was compared with the wet weight. The actual pieces of tissue used in the experiments were too small to make the necessary measurements possible, but an adequate approximation was obtained by determining at each developmental stage the wet and dry weights of a number of whole livers. At each stage sufficient livers were removed from embryos to make up at least 3 mg. wet weight which was determined after removing excess moisture with filter-paper. Corresponding dry weights were obtained after drying for 24 hours over phosphorus pentoxide *in vacuo*. Complete dryness was assured by weighing again after a further period of drying. The dry weight was expressed as a percentage of the wet weight, and the observations repeated a number of times for each of the developmental stages. Results are recorded in Table 1.

RESULTS

The glycogen content of the liver

As described above, glycogen concentration was estimated subjectively. In each liver the region corresponding to that used in the metabolic measurements

TABLE 2

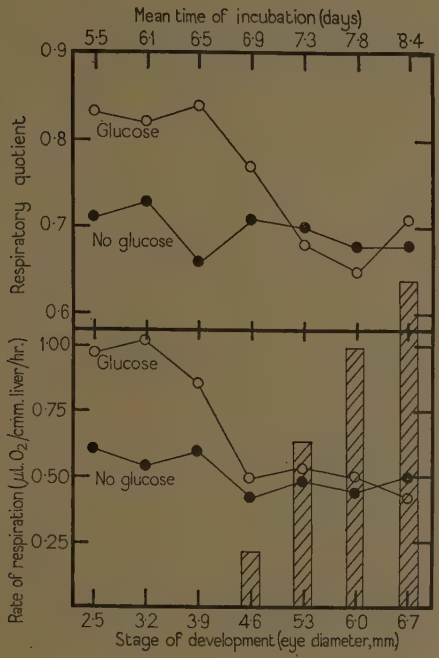
Liver of chicken embryo. Mean values and standard error of the mean at different stages of development for rate of respiration and respiratory quotient of the isolated liver. The measurements were made in media with and without the addition of 0·2 per cent. glucose

Stage of development, eye diameter (mm.)		*Medium with glucose*						*Medium without glucose*					
		R.			*R.Q.*			*R.*			*R.Q.*		
Range	*Median*	*N.*	*M.*	*S.E.*	*N.*	*M.*	*S.E.*	*N.*	*M.*	*S.E.*	*N.*	*M.*	*S.E.*
2·2–2·8	2·5	7	0·98	0·03	7	0·83	0·04	6	0·61	0·07	6	0·71	0·03
2·9–3·5	3·2	6	1·02	0·03	4	0·82	0·03	5	0·54	0·09	4	0·73	0·02
3·6–4·2	3·9	8	0·86	0·09	7	0·84	0·01	6	0·60	0·09	5	0·66	0·02
4·3–4·9	4·6	4	0·49	0·04	4	0·77	0·02	5	0·42	0·01	4	0·71	0·06
5·0–5·6	5·3	5	0·53	0·03	4	0·68	0·06	5	0·49	0·07	4	0·70	0·02
5·7–6·3	6·0	6	0·50	0·01	5	0·65	0·08	4	0·44	0·01	4	0·68	0·02
6·4–7·0	6·7	6	0·42	0·06	6	0·71	0·02	4	0·50	0·03	4	0·68	0·05
10th day	—	5	0·50	0·04	4	0·70	0·02	—	—	—	—	—	—

R. = Rate of respiration, μl. oxygen/c.mm. tissue/hr.
R.Q. = Respiratory quotient.
N. = Number of observations.
M. = Mean.
S.E. = Standard error of mean.

was examined in histological sections and the glycogen concentration assessed for each developmental stage from the examination of 4–8 livers. These assessments are compared by means of the hatched columns in Text-fig. 1. It should

be added that at the stage of eye diameter 3·9 mm. one of five livers showed a trace of glycogen, at stage 4·6 mm. three out of eight were positive, and at 5·3 mm. four out of five. All livers were positive at the later stages represented in Text-fig. 1.



TEXT-FIG. 1. Glycogen content, respiratory rate, and respiratory quotient in the chicken liver during development. The heights of the hatched columns indicate the relative concentrations of glycogen, subjectively assessed in arbitrary units which are not marked on the figure. The measurements of time of incubation and of eye diameter apply to both the upper half of the figure, recording the mean values of the respiratory quotient, and to the lower, recording the mean values of the respiratory rate.

The rate of respiration and the respiratory quotient of the isolated liver

Mean values for each of the developmental stages, together with their standard errors, are recorded in Table 2. The table also includes results obtained from a group of embryos on the tenth day of incubation, when the eye diameter is no longer an adequate indication of differences in the stage of development. In order to relate the variations in respiration and respiratory quotient to one another and to the first appearance of glycogen in the liver cells, the mean values recorded in Table 2 have been reproduced in Text-fig. 1. In both table and figure the results recorded refer to liver tissue from the ventral aspect of the right lobe.

In some cases other regions of the liver were investigated; no significant differences were found.

Some comment is necessary on the results obtained in the medium from which glucose was omitted. Since glycogen is known to break down in the isolated liver it is possible that this process may add sufficient carbohydrate to the medium to affect the results. It can be pointed out, however, that while being prepared for the divers, the portions of liver spent more than 1 hour in the medium with no added glucose. In the course of the necessary manipulations the medium was changed three times, so that it is concluded that any addition of carbohydrate to the medium from liver glycogen would have ceased before the liver tissue was placed in fresh medium in the divers.

DISCUSSION

Glycogen was demonstrated with assurance for the first time in embryos grouped in the developmental stage of eye diameter 4·6 mm. with a mean incubation time of 6·9 days. This time corresponds with the results of Dalton (1937), who, using Best's carmine technique, found that glycogen first appeared in the liver cells of 7-day embryos. There is also agreement with the chemical estimations of Lee (1951), who found measurable amounts of glycogen on the eighth day of incubation.

By reference to Text-fig. 1 it is possible to relate the appearance of glycogen to changes in the rate of respiration of the isolated liver and respiratory quotient, the mean values for which are plotted in the figure. Between eye stages 3·2 mm. and 5·3 mm., while glycogen appears in the liver, there is a decrease in the rate of respiration and in the respiratory quotient when these are measured in the medium containing glucose. The rate of respiration falls from 1·02 to 0·53 μl. oxygen/c.mm. tissue/hr., that is, by about 50 per cent., and the lower rate has not significantly altered by the tenth day of incubation (see Table 2). The respiratory quotient decreases from 0·82 to 0·68 and, as in the case of the respiratory rate, the lower value is maintained subsequently without significant alteration.

To exclude the possibility that the fall in the rate of respiration is due to a dilution of the respiring elements by inert material added during development, the following considerations are advanced. The addition of water could not alone be the cause of a decrease in the respiratory rate of about 50 per cent., because the results set forth in Table 2 show that as the fall in respiration occurs there is no significant alteration in the water content of the liver. Nor, of itself, could the addition of glycogen cause the fall in rate of respiration, for Lee's (1951) observations make it possible to ascertain the approximate amount of glycogen formed in the liver cells in the period during which the fall in respiratory rate occurs. The amount formed is approximately 0·5 per cent. of the wet weight. This constitutes less than 2·5 per cent. of the dry weight, and could not, by dilution of respiring elements, account for the fall of about 50 per cent. in the

rate of respiration. Similar arguments can be used to exclude fat accumulation as the cause of the fall in respiratory rate, if the findings in the guinea-pig embryo can be applied to the chicken embryo. Flexner & Flexner (1950) found that in the first half of gestation the fat content of the liver of the guinea-pig embryo did not rise above 0·14 per cent. of the wet weight.

Text-fig. 1 suggests that the fall in the rate of respiration occurs in livers from embryos at an earlier stage of development than those in which the fall in respiratory quotient was observed. It will be seen, however, that this difference is equivalent to one arbitrary stage of development and to a time interval of 0·4 day, which is of limited significance in view of the standard errors of the mean incubation times set forth in Table 1. Thus the difference in the time of the fall in respiratory rate and respiratory quotient is not inconsistent with both being due to the same metabolic change. This could be the decrease of a metabolic process consuming oxygen and resulting in a respiratory quotient higher than that observed before the changes took place. The breakdown of carbohydrate by a respiratory path, resulting in a respiratory quotient of unity, is such a process.

However, it is necessary to obtain direct evidence that the changes in the rate of respiration and the respiratory quotient are due to a decrease in respiratory carbohydrate catabolism, particularly in view of Needham's (1926*b*, 1932) observations that ammonia is produced in the intact embryo from the combination of oxygen and protein for which the theoretical respiratory quotient is unity. Thus, if any of this ammonia formation took place in the liver of the intact embryo and continued to do so in the conditions of isolation, the decrease in the rate of respiration and the respiratory quotient in the isolated liver might be due to a decrease in ammonia formation.

To obtain direct evidence that the changes are due to alterations in carbohydrate catabolism the rate of respiration and the respiratory quotient of the isolated liver were measured in the absence of glucose from the medium. Before the appearance of glycogen, at the stage of eye diameter 4·6 mm., omission of glucose causes a considerable decrease in the respiratory rate and the respiratory quotient (see Text-fig. 1). These alterations are evidence that before the appearance of glycogen the isolated liver consumes glucose by a respiratory mechanism. On the other hand, after the appearance of glycogen, in developmental stages of eye diameter greater than 4·6 mm., omission of glucose causes no significant alteration of the rate of respiration or the respiratory quotient, from which is concluded that, after the appearance of glycogen, the isolated liver no longer utilizes the oxygen in the catabolism of glucose. This conclusion is supported by the level reached by the respiratory quotient after the appearance of glycogen; the values of 0·65–0·71 make it unlikely that any of the oxygen consumed is utilized in metabolic process resulting in a respiratory quotient of unity. In addition, it will be seen from Text-fig. 1 that, when glucose is omitted from the medium, the respiratory rate and the respiratory quotient before the appearance of glycogen are very nearly the same as those observed after its appearance.

That is to say, omission of glucose from the medium closely reproduces the changes associated with the appearance of glycogen. For this reason it is concluded that decrease in respiratory catabolism of carbohydrate accounts almost entirely for these changes and that there is no important contribution from alterations in ammonia formation or any other metabolic process.

Although the behaviour of the isolated liver is not necessarily the same as its behaviour in the intact embryo, it is nevertheless reasonable to ascribe the changes of metabolism in the isolated liver to changes in enzymatic activity occurring in the intact liver during its development. It is concluded, therefore, that the appearance of glycogen is associated with an alteration of enzymatic activity which at least decreases, and probably destroys, the ability of the liver cells in the intact embryo to break down glucose by respiratory mechanisms. Glycogen formation might, therefore, be ascribed to a diversion of glucose from a catabolic path to one that results in its deposition as glycogen.

In the isolated liver these changes have occurred in the group of embryos included in the stage of development of eye diameter 5·3 mm. which have a mean incubation time of 7·3 days; at this stage, and subsequently, the mean value of the respiratory quotient of the isolated liver ranges from 0·65–0·71 (see Text-fig. 1). It therefore approximates the respiratory quotient resulting from two different metabolic processes: firstly, the respiratory catabolism of fat, which as is well known results in a respiratory quotient of 0·70, and, secondly, the production of uric acid by the combination of protein and oxygen which, as Fiske & Boyden (1926) have shown, has a theoretical respiratory quotient of 0·71. Certain considerations favour the possibility that the latter process accounts for the respiratory quotient found in the isolated liver after the appearance of glycogen and the accompanying disappearance of carbohydrate respiratory catabolism. Firstly, the absence of added protein from the medium does not exclude such a possibility because, if the necessary enzyme systems were present, uric acid formation would be expected to continue, at least for a time, at the expense of intracellular protein. Secondly, if uric acid formation resulted from the oxygen consumption of the liver from 7·3 days of incubation onward, it would correspond to the findings in the intact embryo by Needham (1926*a*) and by Fiske & Boyden (1926). They found that at approximately 7 days' incubation the rate of uric acid formation by the whole embryo increased when related to embryonic body weight. Further, uric acid is a metabolic product of the adult liver, and its formation by the embryonic liver from an incubation time of 7 days onward would be in accordance with the evidence accumulated by Dalton (1937), leading to the conclusion that at the latter part of the seventh day of incubation the hepatic cells become capable of mature function. Finally, the decrease of the respiratory quotient, which suggested the possibility of uric acid formation, has not been observed in the development of a number of other tissues in the chicken embryo. In the midbrain, circulating red blood cells, retina, and in the scleral cartilages the findings *in vitro* are similar; during development the respiratory

quotient remains at unity, indicating that oxygen continues to be used in the catabolism of carbohydrate (O'Connor, 1950, 1952, and unpublished).

SUMMARY

1. In the liver of the chicken embryo the earliest stage at which glycogen was regularly found was a group of embryos of which the eye diameter was 4·3–4·9 mm. The mean incubation time of these embryos was $6·9 \pm 0·14$ days.

2. The appearance of glycogen was associated with changes in the metabolism of the isolated liver in a glucose-containing medium; mean values for the rate of respiration fell from 1·02 to 0·53 μl. oxygen/c.mm. tissue/hr., and the mean values for the respiratory quotient from 0·84 to 0·68. In both cases the lower level had undergone no further significant alteration by the tenth day of incubation.

3. Observations in a medium to which glucose had not been added showed that before the appearance of glycogen omission of glucose caused a decrease in the mean rate of respiration and in the mean value for the respiratory quotient. These changes were not observed after glycogen had appeared.

4. From these changes it is concluded that the appearance of glycogen in the liver of the chicken embryo is associated with the loss of enzymatic activity necessary for the respiratory catabolism of glucose.

5. After the appearance of glycogen in the liver cells the respiratory quotient of the isolated liver suggests that uric acid formation occurs. This possibility has been discussed.

These observations were made while receiving a personal grant from the British Empire Cancer Campaign. Miss Eileen Blake's skilled technical assistance is acknowledged with gratitude.

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Studies on the Development of the Foregut in the Chick Blastoderm

1. The Presumptive Foregut Area

by RUTH BELLAIRS[1]

From the Department of Anatomy and Embryology, University College, London

WITH ONE PLATE

INTRODUCTION

LITTLE attention has hitherto been paid to the early stages in the development of the foregut in the chick. This paper is the first of a series concerned with an investigation into how it develops during the period between the primitive streak stage and the stage of an embryo with about ten pairs of somites. The term 'foregut' refers throughout to the blind diverticulum extending forward into the developing head from the anterior intestinal portal. The present communication opens with a brief consideration of the gross morphological changes which take place; the rest of the paper is concerned with the location in primitive streak and head process stage blastoderms of the presumptive area from which the foregut of the ten somite embryo will develop.

MORPHOLOGY

The following account has been compiled from the study of serial sections of twenty embryos and from the publications of Duval (1889), Adelmann (1922), and Wetzel (1929).

Between the time of laying and the stage when the head process begins to form, the endoderm of the area pellucida lies as a thin sheet of cubical cells below the epiblast. At the borders of the area pellucida it is continuous with the yolky endoderm of the area opaca (Text-fig. 1A). The whole endodermal layer takes part in the general expansion of the blastoderm over the yolk.

The earliest visible changes concerned with foregut formation can be seen at the beginning of the head-fold stage. These changes are as follows:

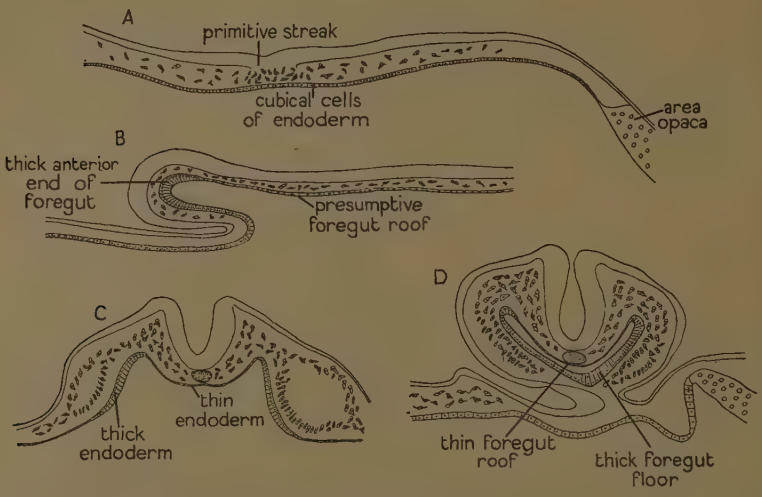
1. *The formation of the most anterior part of the foregut.* This develops at the anterior end of the head process as a wide endodermal diverticulum surrounded by mesoderm; it extends forwards into the developing head and opens posteriorly into the yolk sac (Text-fig. 1B). Its most anterior end and all its floor are thick

[1] *Author's present address*: University Department of Anatomy, Downing Street, Cambridge, England.

[J. Embryol. exp. Morph. Vol. 1, Part 2, pp. 115–24, June 1953.]

and consist of columnar epithelium, but its roof gradually becomes thinner behind the anterior thickened zone.

2. *Thickening of the endoderm on either side of the head process.* The thick floor of the anterior diverticulum is continued posteriorly as a thickened region



TEXT-FIG. 1. Diagrams representing various stages in the development of the foregut. A, C, and D are directly from specimens. B is after Duval and Adelmann.

A. Primitive streak stage: transverse section across primitive streak. The area pellucida endoderm consists of cubical cells.

B. Head fold stage: longitudinal section of developing head fold. The anterior end and floor of the developing foregut are of columnar epithelium: the presumptive roof is mainly squamous.

C. Early embryonic stage: transverse section posterior to the head fold. The endoderm lying beneath splanchnic mesoderm is thickened; the endoderm lying beneath the developing somites and notochord is thin.

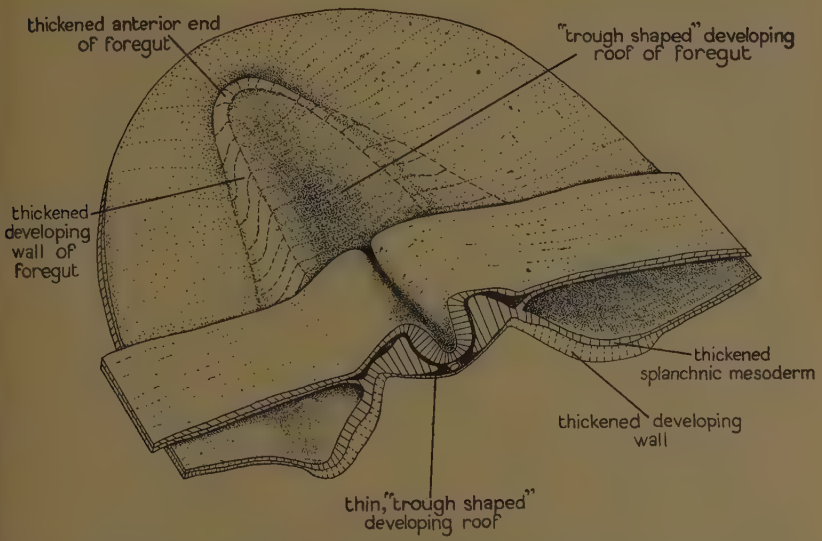
D. Embryonic stage: transverse section across formed foregut. The floor is thick and columnar; the roof is thin and squamous.

on either side of the head process. Later this thickening gradually spreads backwards in the endoderm lying beneath the splanchnic mesoderm. The endoderm here becomes about twice as thick as that which lies beneath the somites and notochord (Text-figs. 1C and 2). The cells become large and arranged in a columnar epithelium, the nuclei tending to lie on the mesodermal side. This thick epithelium becomes even thicker as development proceeds.

3. *The assumption of a dorsally concave, 'trough-like' form by the endoderm lying medially between the two thickened regions* (Text-figs. 1C and 2). The cells here, which were formerly cubical, now become squamous with flattened and widely separated nuclei.

As the formation of the embryonic axis proceeds the thickened parts of the

endoderm swing ventro-medially and meet in the midline where they fuse to form the backward continuation of the floor of the foregut (Text-figs. 1C, 1D, and 3). At this junction of tissues contact of the foregut floor with the adjacent



TEXT-FIG. 2. Diagram of the endoderm in the early embryo. The anterior half of the area pellucida is represented as if seen from above after removal of most of the ectoderm and mesoderm. The areas outlined by broken lines indicate the extent of the thickened endoderm.

TEXT-FIG. 3. Diagram representing a T.S. across the developing foregut region. On the left side the thickened region is swinging ventro-medially to form the foregut floor. On the right side the corresponding part of the endoderm, already thicker, is represented as having almost reached the midline. The interrupted lines represent stages in the displacement of the future foregut floor, the arrows the direction of the movement.

extra-embryonic endoderm is lost and the latter forms the endodermal roof to the yolk sac. This formation of the foregut floor begins anteriorly and gradually spreads backwards. The floor is thick whilst the roof remains thin. The foregut as a whole is dorso-ventrally flattened, but the floor is concave dorsally like the already 'trough-shaped' roof. It retains this characteristic appearance until the stage of about 15–20 pairs of somites, when the first steps occur in the regional differentiation of the gut.

The movements by which the future floor of the foregut is brought medio-

ventrally also serve to bring the associated splanchnic mesoderm into a corresponding medial and ventral position. This close association of the two layers has been emphasized in a recent paper by Rudnick (1952).

THE PRESUMPTIVE FOREGUT AREA

Method

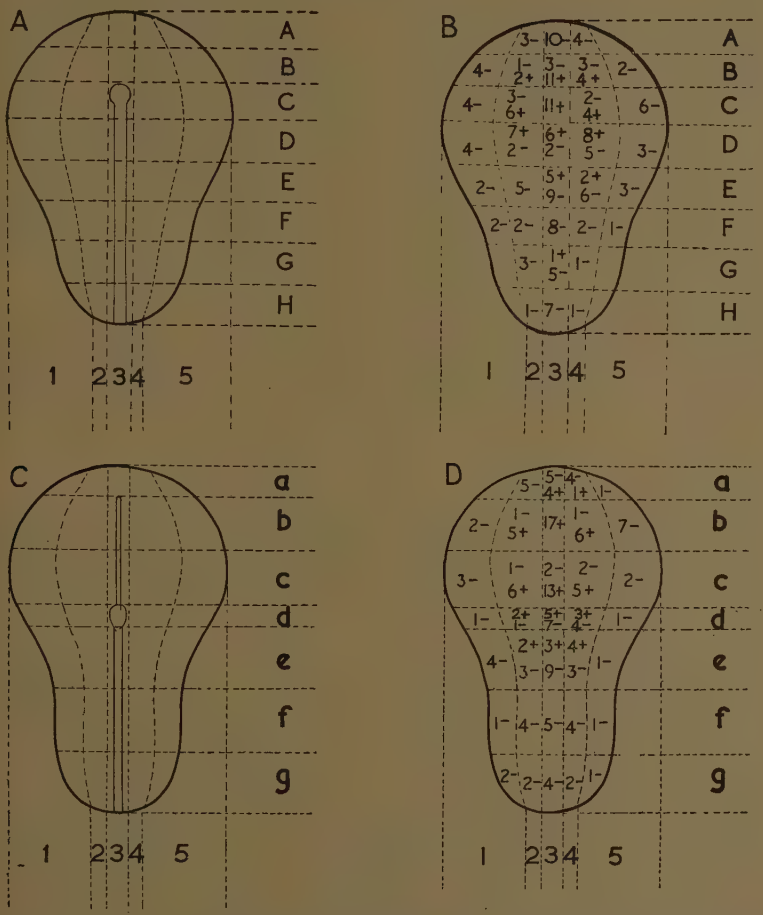
Maps showing the location of the presumptive areas of the epiblast have been presented by Wetzel (1929), Gräper (1929), Pasteels (1937), and Spratt (1952). They were prepared either entirely, or partially as in the case of Spratt's, from the results of vital marking experiments carried out upon the blastoderm lying *in situ* above the yolk. The inaccessibility of the endoderm in its normal situation precludes the possibility of using the same technique to determine the position of the presumptive foregut. In all cases therefore, in the present investigation, vital marking was carried out upon blastoderms which had been removed from the yolk and explanted on a plasma clot (technique of Waddington, 1932). Specimens were placed with the epiblast surface against the clot and the endoderm uppermost. Vital dyes were not used because of the difficulty, notorious in the chick, of retaining the colour throughout the preparation of serial sections. Instead finely powdered carbon (animal charcoal) was put on a selected region of the endoderm to act as a mark.

In the present work I found it advantageous to remove as much saline as possible from the exposed tissue before applying the mark, otherwise the carbon tended to float off. As reported by Spratt (1946) for the epiblast layer, the dry carbon adheres to the surface of the cells most tenaciously. Jacobson (1938) placed drops of Indian ink on the endodermal surface of blastoderms and reported phagocytosis of the carbon particles. In the present experiments also, small carbon particles were sometimes found within the cytoplasm of the endoderm cells, though the position of the ingested material usually corresponded well with that of the main body of the mark itself (Plate, fig. B). Only small patches of carbon were used as marks (about 5μ to 50μ in diameter). Larger masses tended to fall through the endoderm and become adherent to the mesoderm (Jacobson, 1938).

Waddington (1950) has objected to the use of carbon as a marker because it is 'by no means certain that the solid particles always remain attached to the cell'. Because of this criticism I have performed large numbers of experiments and these have given fairly consistent results. Had the carbon been moved independently of the cells somewhat erratic answers might have been expected. It is improbable that the marking technique had any toxic influence on the embryo, for no difference could be detected between the development of experimental cultures and of unmarked control blastoderms.

After each specimen had been marked it was drawn to scale and the position of the carbon patch plotted. After further incubation for 24 to 36 hours the embryo was redrawn and, where it was visible, the position of the carbon was

again noted (Plate, fig. A). The embryos were subsequently fixed in Bouin's fluid, sectioned at 10μ, stained with Weigert's haematoxylin, and examined for traces of carbon in the foregut (Plate, fig. C).



TEXT-FIG. 4. Marking experiments.

A and C. The arbitrary regions which have been distinguished in the endoderm at the long streak and head process stages respectively.

B and D. The results obtained at the long streak and head process stages respectively. The number of specimens in which inclusion in the foregut of a marked group of cells was (+) and was not (−) obtained is indicated by the numerals in each region.

Marking experiments have been carried out upon blastoderms at two stages, the long or definitive primitive streak stage and the head process stage, as defined by Waddington (1932) and Abercrombie (1950).

To make it possible to describe and compare the marks in different blastoderms accurately, a number of arbitrary regions were distinguished in each specimen (Text-fig. 4, A and C). The variation in size between individuals is considerable at these stages, so direct measurements have been avoided for fixing the extent of each region. Morphological landmarks were employed where possible; otherwise the regions were assessed as fractions of the total width or length of the area pellucida. Thus the long streak stage blastoderm was considered as consisting of five longitudinal strips: strip *3* enclosed the primitive streak: strips *2* and *4* were the regions on either side, and they extended half of the way to the area opaca: strips *1* and *5* were the lateral parts of the area pellucida (Text-fig. 4A).

These strips were in their turn considered as being subdivided into six regions across the primitive streak of equal antero-posterior length (*C*, *D*, *E*, *F*, *G*, and *H*) and two equal ones (*A* and *B*) anterior to the primitive node. In this way the area pellucida of the long streak stage was treated as forty different parts.

Similarly the head process stage (Text-fig. 4C) was considered as possessing five longitudinal strips assessed in a comparable manner, which were subdivided transversely into seven regions. Level *a* lay anterior to the head process; levels *b* and *c* each contained half of the head process; level *d* included the primitive node; levels *e*, *f*, and *g* each incorporated a third of the primitive streak.

Results

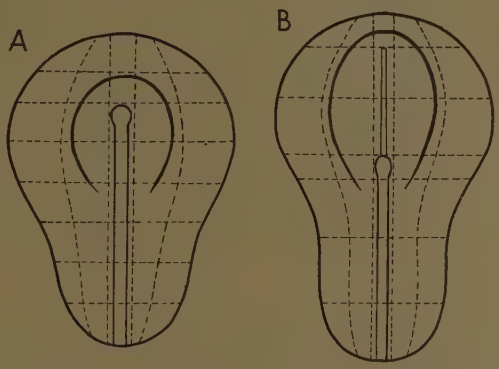
The results of the marking experiments are summarized in Text-fig. 4, B and D. Taking region *C4* in Text-fig. 4B as an example, the convention used is as follows: 4 + means that in four specimens the carbon mark, when placed in this position, became included in the foregut; and 2 – means that in two specimens the mark did not become included in the foregut.

In the *long streak* blastoderms it will be noticed that the positive results are distributed around the anterior end of the primitive streak. In no case were laterally situated marks in strips *1* and *5* at any level enclosed in the foregut. In the regions close to, and on either side of, the anterior end of the primitive streak (i.e. regions *2* and *4* of *B*, *C*, *D*, and *E*) 33 from a total of 60 marks became enclosed in the gut. Most of the negative results were obtained from specimens in which marks were placed at the lateral edges of these regions and in these the marked cells were usually found eventually in the yolk-sac endoderm. Carbon placed more medially in regions *2* and *4* usually became included in the foregut. The occurrence in a given region of positive results from some specimens and negative ones from others may have been due in part to experimental error in fixing the boundaries of the regions, but it is also likely that the lateral extent of the presumptive area varies according to the specimen.

A similar variation from individual to individual may also be a feature of the posterior end of the presumptive area, for here too the positive and negative results overlap. To some extent, however, the fate of a marked group of cells depended on the amount of development which had occurred in that embryo before fixation. It is likely, for example, that in the two negative specimens shown

in *D3* the marked cells would have become part of the foregut had development proceeded further. In regions *D* and *E*, however, other types of individual differences also affected the results.

Firstly, there was sometimes a backward or sideward movement of cells. Fourteen specimens were marked in region *E3*, but the carbon was subsequently found in the foregut of five embryos only. In 8 of the other 9 cases the mark was discovered at the hind end of the primitive streak, i.e. the marked cells had migrated posteriorly. In regions *E2* and *4*, of the 11 marks which did not become



TEXT-FIG. 5. The presumptive foregut area indicated by carbon marking experiments. A. The long streak stage. B. The head process stage.

included in the foregut, 4 were found to have migrated laterally and 2 to have moved posteriorly. In three other specimens a second circumstance connected with the non-inclusion of midline marks in regions *D* and *E* may be noted; marks placed in position *E2* or *E3* were subsequently found on the yolk-sac endoderm in the midline ventral to the heart. The carbon extended forward for 20μ from the level of the anterior intestinal portal.

Text-fig. 5A shows the region in which positive results were usually obtained at the long streak stage, i.e. the presumptive foregut area. Although for convenience precise limits are shown, the borders of the presumptive foregut in any given blastoderm may not coincide exactly with these boundaries.

In *head process stage* blastoderms the results obtained from marking are summarized in Text-fig. 4D. The distribution of the positive results is similar to that obtained at the long streak stage. The lateral extent is comparable: in regions *2* and *4* of levels *a, b, c, d,* and *e*, 34 out of a total of 59 marks became enclosed in the foregut. Although the positive results appear to extend farther anteriorly in the head process than in the long streak stage, the region of positive results is morphologically comparable at the two stages in that it reaches to about the most anterior limit of the invaginated mesoderm (Adelmann, 1922; Wetzel,

1929). Posterior migration of the medially situated marks occurred as in the long streak stage, e.g. of 12 specimens marked at *e3*, the marks became enclosed in 3, moved posteriorly in 6, laterally in 2, and remained apparently in the same place in the remaining embryo. Of the 8 blastoderms marked in regions *f2* and *f4*, 5 showed a migration in a postero-lateral direction.

Text-fig. 5B shows the area in which positive results were obtained, i.e. the presumptive foregut region at the head process stage.

DISCUSSION

Rudnick & Rawles (1937) have investigated the ability of isolated fragments of blastoderms to develop gut when cultured upon the chorio-allantois. After 8 to 10 days many of their specimens had grown into tissues which could be recognized histologically as different levels of the gut, e.g. small or large intestine. The time during which blastoderms may be maintained in tissue culture by the method I have used is much shorter and differentiation does not proceed so far. There is no evidence in my results to show which part of the simple foregut tube will give rise to a particular region, such as the oesophagus, or even to indicate if a certain level of future adult gut was yet included in the foregut when the experiment was terminated at the time of fixation. It may well be that the small intestine lies farther posteriorly than the presumptive area shown in my maps. Despite these differences in technique, however, it is interesting to compare the endodermal potencies of fragments on the chorio-allantois with the results of the present marking experiments.

Using the long streak stage Rudnick & Rawles (1937) divided the blastoderm into four portions. From each of these gut was subsequently obtained. According to my results, each of their pieces contained a fragment of the presumptive foregut area.

With the head process stage, however, Rudnick & Rawles obtained very few cases of gut (about 6 per cent.) from isolates taken from the 'anterior embryonic and node fields', i.e. the region which is presumably equivalent to *a*, *b*, *c*, and *d* in Text-fig. 4C. In grafts from the streak field, however (i.e. my regions *e*, *f*, *g*, and *h*?), they obtained 'organized gut' in 20 per cent. of their isolates. In the posterior parts of this field gut formed in median isolates only, whereas in the level 0·3–0·7 mm. behind the pit, lateral pieces as well had this potency.

The failure of Rudnick & Rawles to obtain more than a few cases of gut from the 'anterior embryonic and node field', that is from the very heart of the presumptive area, is perhaps significant, for ordinarily the potency region for a given tissue may be expected to include its presumptive area. If, however, certain parts of the presumptive area fail to differentiate when isolated (i.e. are not included in the region of potency for that particular tissue), the lack of some condition necessary for their differentiation is indicated. Rudnick & Rawles report that the pharynx and oesophagus were never found as organs. It may be that these structures normally develop in the 'anterior embryonic and node field'

under the influence of neighbouring tissues. The results of Rudnick & Rawles are at least highly suggestive of some difference existing between the head process and the anterior end of the primitive streak in head process stage blastoderms which affects the ability of the foregut to differentiate under the conditions of chorio-allantoic grafting.

It has frequently been suggested that the development of the gut from undifferentiated endoderm is dependent on the presence of mesoderm (Rudnick & Rawles, 1937; Hunt, 1937; Rudnick, 1944; Waddington, 1952). Despite the valuable marking experiments of Gräper (1929), Wetzel (1929), Pasteels (1937), and Spratt & Condon (1947), the distribution of mesoderm at these stages is not fully understood. It would not be out of place, however, to emphasize here the close correspondence which exists between the distribution of the presumptive foregut and the presumptive neural plate (Pasteels, 1937; Spratt, 1952). Neural plate is believed to be induced by the underlying mesoderm (Waddington, 1932, 1933); and it may well be that this mesoderm performs a double function by also influencing the development of subjacent endoderm. This concept of foregut induction will be discussed more fully in a subsequent paper.

SUMMARY

1. A brief morphological account is given of the changes which take place in the endoderm during the first stages of foregut formation. The period of development concerned is from the stage of the primitive streak to that of the embryo with about ten pairs of somites.

2. Experiments are described in which the endoderm of chick blastoderms grown *in vitro* was marked with carbon particles. By this means the presumptive foregut area was found to lie around the anterior end of the primitive streak in the long streak stage, and around the head process and anterior end of the primitive streak at a slightly later stage of development.

I am most grateful to Mr. M. Abercrombie for his critical interest in this work and to Mr. H. Barker for his technical advice. I am indebted to my husband, Dr. A. d'A. Bellairs, for Text-fig. 2, to Miss Joyce Hubbard for Plate, fig. A, and to Mr. J. A. F. Fozzard, F.R.P.S., for Plate, fig. C.

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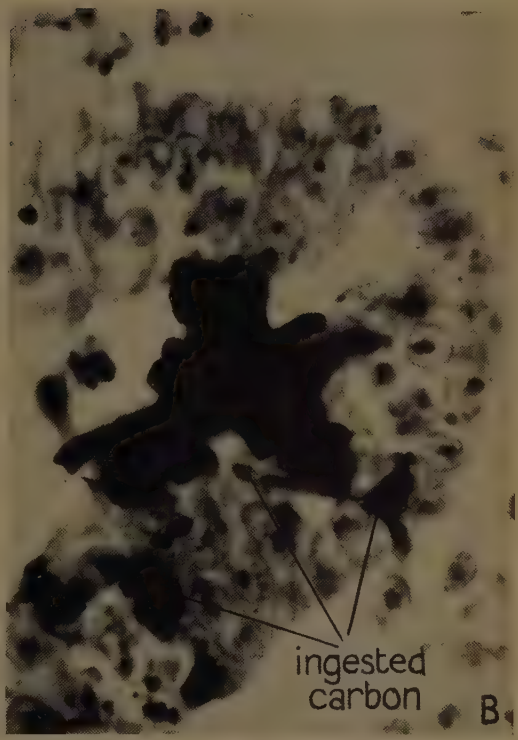
EXPLANATION OF PLATE

FIG. A. A specimen fixed 24 hours after marking with carbon particles. Two carbon marks, which did not become enclosed in the foregut, can be seen on the yolk-sac endoderm.

FIG. B. Carbon particles ingested into the endodermal cells; they are closely associated with the main part of the carbon mark.

FIG. C. Transverse section across the embryo shown in fig. B. The carbon is in the floor of the foregut. The neural plate is somewhat distorted, because the blastoderm was explanted with its dorsal surface against the clot.



R. BELLAIRS
Plate 1

Les Effets de la centrifugation sur la blastula et la gastrula des Amphibiens

II. Étude comparative de la sensibilité en fonction des stades et des espèces

par J. PASTEELS[1]

Université de Bruxelles, Laboratoire d'Embryologie de la Faculté de Médecine

AVEC PLANCHES 1–3

INTRODUCTION

DANS le premier mémoire de cette série nous avons analysé le mécanisme par lequel des organes surnuméraires ou des axes embryonnaires secondaires peuvent apparaître aux dépens de l'ectoblaste, à la suite d'une centrifugation de la blastula ou de la gastrula.

Il s'agit, rappelons-le, d'une activation directe de l'ectoblaste dont le plein rendement se manifeste chez la Grenouille rousse (*Rana temporaria*) lorsque les œufs ont été centrifugés aux stades de la blastula avancée ou de la jeune gastrula. Cependant, dans d'autres conditions, soit qu'il s'agisse d'œufs de Grenouille centrifugés à d'autres stades, ou encore d'œufs d'une autre espèce, comme ceux de l'Axolotl, cette activation n'est que subliminale et ne peut s'exprimer qu'après contact préalable avec l'organisateur normal.

Il apparaît ainsi que pour une espèce déterminée, la sensibilité du germe est spécifique d'une phase assez limitée du développement; que suivant le stade auquel les œufs sont centrifugés les réactions de l'ectoblaste présentent des variantes non seulement quantitatives, mais aussi *qualitatives*; que lorsqu'on s'adresse à des œufs d'Amphibiens provenant de différentes espèces, les réponses peuvent être diverses.

Les variations chronologiques de la sensibilité du germe au cours de la morphogenèse primaire sont d'un intérêt capital: elles témoignent de l'évolution progressive et irréversible des prodromes morphogénétiques que recèle le germe encore dépourvu de forme. Aussi aucun moyen d'analyse qui permet de déceler cette évolution ne peut être négligé.

Nous ajouterons, sans vouloir trop anticiper, que l'étude systématique que nous allons poursuivre actuellement, nous entraînera, pour un stade déterminé

[1] *Author's address*: Laboratoire d'Embryologie, Faculté de Médecine de l'Université, 97 Rue aux Laines, Bruxelles. Belgium.

[J. Embryol. exp. Morph. Vol. I, Part 2, pp. 125–45, June 1953]

également, à déceler une transformation du chordomésoblaste et de son pouvoir inducteur.

Une étude systématique des résultats de la centrifugation depuis le stade morula jusqu'à la gastrula avancée a donc été poursuivie chez 9 espèces d'Amphibiens. Ce sont ces résultats qui font l'objet du présent mémoire.

Les expériences ont consisté, comme nous l'avons dit dans la première partie, en une centrifugation modérée (=460 g.) pendant des temps variant de 10 secondes à 10 minutes. Elles ont été réalisées systématiquement, à tous les stades présumés sensibles, chez les espèces suivantes:

Anoures: *Rana temporaria.*
Xenopus laevis.
Discoglossus pictus.
Urodèles: *Siredon mexicanum* (Axolotl).
Pleurodeles waltii.
Ambystoma punctatum.
Triturus alpestris, T. helveticus, et *T. vulgaris.*

Autant que possible, un nombre suffisant d'embryons a été traité, fixé et examiné sur coupes pour permettre une comparaison valable des résultats. On en trouvera le détail au cours de l'exposé des résultats.

Les expériences faites sur l'espèce américaine *Ambystoma punctatum* ont été réalisées au cours d'un séjour (en 1948) à l'Osborn Zoological Laboratory, Yale University. Nous tenons à exprimer tous nos remerciements au Professeur J. S. Nicholas qui nous a accueilli dans son laboratoire, ainsi qu'au Dr. W. W. Ballard qui nous a récolté et envoyé des pontes en provenance du New Hampshire.

RÉSULTATS. I. ETUDE COMPARATIVE DES RÉSULTATS DE LA CENTRIFUGATION AUX DIFFÉRENTS STADES CHEZ *RANA TEMPORARIA*

C'est chez la Grenouille rousse que nous avons pu disposer des pontes les plus abondantes. Nous avons ainsi pu comparer la sensibilité des œufs prélevés à différents stades de la même ponte et comparer également les effets obtenus sur des pontes différentes. Comme il s'agit d'une espèce particulièrement sensible, où les différences selon les stades traités sont très accusées, les résultats obtenus devront être exposés en détail.

Pour établir la chronologie des stades chez la Grenouille rousse, nous nous reportons, comme nous l'avons fait ailleurs (Pasteels, 1945) à des élevages à 20°. (Des expériences complémentaires ont montré que dans les limites physiologiques normales, des changements de température n'affectaient pas les résultats de la centrifugation.) Rappelons (cf. loc. cit., Tableau II, p. 6) que dans ces conditions le stade de la très jeune gastrula est atteint à la 21e heure après fécondation. La phase de sensibilité de l'œuf de Grenouille s'étend de la 15e heure (blastula moyenne) à la 25e (blastopore en faucille). Elle présente de petites variantes d'amplitude suivant les pontes. D'ailleurs, même au moment de la sensibilité

optimale (21e heure), certaines pontes réagissent avec plus de facilité que d'autres.

L'étude systématique, de stade en stade, a été faite sur un nombre suffisant d'embryons, au cours de cinq expériences; ce nombre et les stades envisagés sont dans le Tableau 1.

1. *Analyse d'une expérience type.*

Examinons à titre d'exemple les résultats obtenus au cours de l'expérience 1945/2 (cf. Tableau 1).

(*a*) La première centrifugation a été faite 15 h. après fécondation (20°), les œufs étant au stade de la blastula moyenne. 145 œufs ont été fixés, dont 104 ont été reconnus normaux, tandis que 41 (donc 27 pour cent) montraient des anomalies.

Celles-ci rentrent sans exception dans le cadre de la 'sensibilisation subliminale' (cf. Pasteels, 1953, p. 18). Rappelons qu'il s'agit dans ce cas d'organes

TABLEAU 1

Heures de développement (à 20°)	*Nombres d'embryons étudiés*				
	Exp. 1941/1	*Exp. 1941/3*	*Exp. 1945/2*	*Exp. 1945/3*	*Exp. 1945/6*
14 h.	—	—	—	130	—
15 h.	94	—	145	—	66
16 h. 30	116	—	—	125	—
17 h.	—	—	166	—	68
18 h.	105	—	—	—	—
18 h. 30	—	—	—	104	—
19 h.	—	—	73	—	84
19 h. 30	116	—	—	—	—
21 h.	34	36	61	75	70
22 h. 30	31	37	—	—	—
23 h.	—	—	67	—	—
24 h.	—	100	—	—	—
25 h.	—	—	85	—	100

surnuméraires toujours associés étroitement au névraxe primaire. Chez la Grenouille rousse, ces anomalies se manifestent par des dédoublements partiels et irréguliers des cavités cérébrales, avec des inclusions de tissu chordal au milieu du tissu nerveux; l'otocyste est souvent hypertrophié, tandis que de petits otocystes surnuméraires peuvent se manifester à des endroits variés.

Les fig. A, B, C de la Planche 1 nous en donnent quelques exemples obtenus dans ce même lot. La fig. A montre l'existence d'un amas irrégulier de cellules chordales dans une cloison clivant anormalement la partie dorsale du diencéphale. Les fig. B et C présentent des coupes successives à travers un rhombencéphale; sur la première, le rhombencéphale est dédoublé; sur la seconde le tissu rhombencéphalique en excès est presque entièrement remplacé par un amas très irrégulier de tissu chordal.

Nous noterons également sur ces deux dernières figures une caractéristique

fréquente des embryons de Grenouille centrifugés à ces jeunes stades: *l'extrémité antérieure de la chorde dorsale est fortement hypertrophiée.*

Le sommaire des résultats obtenus à ce premier stade peut se résumer par le Tableau 2 (en l'absence d'élaboration statistique, les pour cent calculés ne représentent qu'un ordre de grandeur).

TABLEAU 2

Résultats de la centrifugation 1945 / 2, 15 h. après fécondation

I. Embryons étudiés: 145.
Embryons anormaux: 41 (27%).
II. Anomalies du système axial primaire (% calculés sur le total de 41):
Dilatation antérieure de la chorde primaire: 7 (17%).
Plis ou dédoublements partiels du prosencéphale: 22 (53%).
Plis ou dédoublements partiels du rhombencéphale: 27 (66%).
Dédoublements partiels de la moelle: 2 (5%).
Hypertrophie d'un otocyste primaire: 19 (47%).
Nodules chordaux dans le névraxe: 8 (19%).
III. Systèmes indépendants de l'axe primaire:
Otocystes et dérivés ganglionnaires en excès, mais en position anormale: 3 (7%).
Glandes adhésives, idem: 2 (5%).

(*b*) Le second lot de cette expérience a été centrifugé 17 h. après la fécondation. Il s'agit de blastules moyennes. La fréquence des diverses anomalies est devenue plus grande (cf. Tableau 3). Les résultats obtenus sont de même nature que les précédents. Notons cependant une tendance des systèmes organiques secondaires à devenir indépendants des axes primaires. Par exemple, nous voyons sur la fig. D de la Planche 1 le curieux aspect d'un nodule chordal, encore pédiculisé dans l'ectoblaste dont il dérive, faisant saillie à la voûte d'un 'cerveau', d'ailleurs très irrégulier. Plus en arrière (Planche 1, E) ce nodule se prolonge par une chorde, devenue autonome, qui touche le pharynx au niveau duquel elle a induit un diverticule de la voûte.

Cette tendance se manifeste aussi par l'apparition plus fréquente, à des endroits divers de la paroi ventrale de l'embryon, de nodules où l'ectoblaste s'organise en masses neuroïdes, avec formations ganglionnaires, otocystes, parfois des glandes adhésives. Il s'agit toutefois toujours de systèmes 'mineurs', sans aucune différenciation neurale vraie.

La dilatation de la chorde se présente avec la même fréquence que dans le lot précédent.

En bref, les résultats de ce lot de 17 h. se résument par le Tableau 3.

(*c*) Le troisième lot concerne des œufs centrifugés à la 19^{e} heure (20°) après la fécondation, donc au stade de la blastula avancée. Cette fois-ci *tous* les œufs présentent des anomalies. 73 d'entr'eux (pris au hasard) ont été fixés et étudiés sur coupes. Chez *tous* on observe des cavités supplémentaires dans le prosencéphale, le myélencéphale, ou les deux à la fois, le plus souvent (58 cas sur 75) entremêlées de nodules chordaux. Ces manifestations, que nous avons qualifiées

précédemment de 'sensibilisation subliminale' augmentent en fréquence au point d'être devenues absolument générales.

TABLEAU 3

Résultats de la centrifugation 1945/2, 17 h. après fécondation

I. Embryons étudiés: 166.
Embryons anormaux: 81 (48%).
II. Anomalies du système axial primaire (% calculés sur le total de 81):
Dilatation antérieure de la chorde primaire: 15 (18%).
Plis ou dédoublements partiels du prosencéphale: 60 (74%).
Plis ou dédoublements partiels du myélencéphale: 57 (70%).
Dédoublements partiels de la moelle: 6 (7%).
Hypertrophie d'un otocyste primaire: 37 (48%).
Nodules chordaux dans le névraxe: 28 (39%).
III. Systèmes indépendants de l'axe primaire:
Otocystes, ou masses neuroïdes, ou ganglionnaires en position atypique: 13 (18%).
Glandes adhésives, en position atypique: 11 (13%).

La dilatation antérieure de la chorde dorsale se manifeste dans 19 cas sur 73, la fréquence en est donc un peu accrue.

Mais le changement décisif qui apparaît par rapport au stade précédent, c'est la manifestation — dans 64 de ces 73 embryons — d'axes secondaires. Les cavités rhombencéphaliques que nous avons vues annexées au cerveau primaire, s'en détachent plus nettement, s'accompagnent de chorde, parfois de muscles, se prolongent par une moelle — et ainsi se constitue un axe secondaire autonome sur le flanc ou le ventre de l'embryon.

Deux caractéristiques de ces axes méritent d'être relevées. La première, c'est leur caractère exclusivement chordencéphalique ou spinal. *Jamais*, nous n'observons d'organes acrencéphaliques ('archencéphaliques' suivant F. F. Lehmann) autonomes, indépendants du cerveau primaire.

La seconde, c'est la structure très incoördonnée de ces axes. A titre d'exemple nous examinerons la fig. F de la Planche 1 et les fig. A–C de la Planche 2. Sur la fig. F de la Planche 1 nous voyons le côté du cerveau primaire se prolonger par une vaste cavité à allure rhombencéphalique. Au niveau de la voûte de ce quatrième ventricule, fait hernie une grosse masse de tissu chordal. Ce rhombencéphale se contourne, se prolonge par un tube nerveux, d'abord atypique (coupé au-dessus de la chorde sur cette figure), puis par une moelle accompagnée de chorde, s'engageant dans une véritable nageoire secondaire. Le début de cette nageoire, où l'épiblaste est soulevé par du mésenchyme, se voit également sur la fig. F. Forme et rapports de la chorde et du névraxe sont aberrants, il n'y a ni muscles ni pronéphros.

L'exemple de la fig. A de la Planche 2 (avec des images de détail sur coupes successives sur les fig. B et C) est encore plus curieux car il témoigne d'une *véritable hésitation* — d'un type fréquent — entre les différenciations neurale et chordale. Sur le côté du bulbe était apparue une expansion à caractère rhomben-

céphalique. Mais voilà qu'elle fait place (Planche 2, fig. A et B) à un mélange inextricable, de tissu nerveux (où l'on distingue un début de moelle) et de tissu chordal, le tout s'accompagnant dorsalement d'un petit amas de cellules musculaires. Plus en arrière (fig. C), au sein même de ce qui paraissait être une moelle — et qui en a encore la lumière — les cellules se vacuolisent et forment ainsi de la chorde.

Ce manque d'équilibre entre les organes axiaux se caractérise surtout, dans le lot que nous envisageons actuellement (blastula avancée), par une prépondérance de la chorde sur les myotomes. La chorde a été reconnue en effet dans *tous* les axes secondaires (64 cas), tandis que des cellules musculaires — et encore en petite quantité — ne se montrent que dans cinq cas; des tubes pronéphritiques n'ont jamais été observés.

Les résultats de cette troisième centrifugation sont résumés sur le Tableau 4, que l'on pourra comparer utilement aux Tableaux, 2 et 3.

TABLEAU 4

Résultats de la centrifugation 1945/2, 19 h. après fécondation

I. Embryons étudiés: 73.
Embryons anormaux: 73 (100%).
II. Anomalies du système axial primaire:
Dilatation antérieure de la chorde primaire: 19 (26%).
Plis ou dédoublements partiels du prosencéphale ou du rhombencéphale: 73 (100%).
Nodules chordaux dans le cerveau primaire: 58 (79%).
Hypertrophie d'un otocyste primaire: 27 (37%).
III. Systèmes axiaux secondaires:
Total: 64 (88%).
à caractère nuqual uniquement: 24 (33%).
à caractère nuqual et troncal: 40 (55%).
Ces systèmes axiaux contiennent:
du névraxe: 64 cas (88%).
de la chorde: 64 cas (88%).
des myotomes: 5 cas (7%).
des otocystes: 27 cas (37%).
des dérivés de crête neurale: 26 cas (36%).
de la glande adhésive: 35 cas (48%).

(*d*) Le quatrième lot a été centrifugé à la 21e heure, c'est-à-dire au moment de l'apparition de la toute première encoche blastoporale. Ici aussi tous les embryons réagissent. A première vue, les résultats sont identiques à ceux de la série précédente. Notons cependant quelques différences notables (basées sur l'étude de 61 embryons).

La chorde primaire est toujours normale: en aucun cas, on n'observe une dilatation de sa partie antérieure. Les dédoublements partiels du cerveau, contrairement à ce qui se passait dans tous les lots précédents, ne présentent plus d'inclusions chordales. Les axes secondaires autonomes ont augmenté de fréquence, et de fait se rencontrent dans tous les cas, à deux exceptions près. Leur nature a toutefois changé: le névraxe y prédomine, mais la chorde y est devenue

plus rare (22 pour cent des cas seulement), en revanche les myoblastes sont devenus plus fréquents (50 pour cent), et dans 28 pour cent des cas, les ébauches secondaires s'accompagnent de tubes pronéphritiques, avec un uretère primaire éventuel. Or, dans la série précédente, aucune formation rénale ne s'était manifestée. Un exemple d'axe secondaire obtenu à ce stade, avec moelle, beaucoup de myotomes, peu de chorde, et un pronéphros, se voit sur la fig. D de la Planche 2.

Le sommaire de ces résultats est exposé sur le Tableau 5.

TABLEAU 5

Résultats de la centrifugation 1945/2, 21 h. après fécondation

I. Embryons étudiés: 61.
Embryons anormaux: 61 (100%).
II. Anomalies du système axial primaire:
Dilatation antérieure de la chorde dorsale: 0.
Plis ou dédoublements partiels du prosencéphale ou du rhombencéphale: 60 (98%).
Nodules chordaux dans le cerveau primaire: 0.
Hypertrophie d'un otocyste primaire: 15 (25%).
III. Systèmes axiaux secondaires:
Total: 59 (98%).
à caractère nuqual uniquement: 16 (26%).
à caractère nuqual et troncal à la fois: 41 (67%).
Ces systèmes axiaux contiennent:
du névraxe: 59 cas (100%).
de la chorde: 13 cas (21%).
des myotomes: 30 cas (49%).
du pronéphros: 23 cas (38%).
des otocystes: 37 cas (61%).
des dérivés de crête neurale: 39 cas (64%).
de la glande adhésive: 17 cas (27%).

(*e*) Un cinquième lot de la même expérience a été centrifugé à la 23e heure (lèvre dorsale du blastopore bien marquée). Quantitativement et qualitativement, un changement profond apparaît à présent. Sur 67 embryons examinés, 28 seulement présentent des anomalies. La moitié de ceux-ci montrent des cavités supplémentaires dans le cerveau primaire. Les complexes organiques autonomes apparaissent dans 23 cas, mais 9 d'entr'eux seulement peuvent être qualifiés d''axes secondaires'. Encore ces axes sont-ils très imparfaits, réduits le plus souvent à leur composante neurale (rhombencéphale suivi d'une petite moelle); on n'y observe *jamais* de chorde, ni de pronéphros, et des somites dans trois cas seulement. Tous les autres complexes organiques secondaires sont mineurs, constitués d'otocystes associés souvent à des ganglions et une petite nageoire soulevée par du mésenchyme (il s'agit donc de dérivés de la crête neurale); les glandes adhésives ne se sont montrées que deux fois (cf. Tableau 6). Les petites queues secondaires s'accompagnent souvent d'un proctodaeum.

(*f*) Enfin, une dernière centrifugation a été réalisée à la 25e heure (blastopore en croissant). Sur 85 embryons étudiés, 9 seulement ont réagi. Les modifications

de l'axe primaire se bornent à deux cas: une hypertrophie d'un otocyste, et un dédoublement du rhombencéphale. Quant aux systèmes indépendants, ils sont surtout formés par des amas de mésenchyme et de ganglions, dans un seul cas on trouve une formation rhombencéphalique, dans deux cas une petite moelle. Comme on pouvait s'y attendre, d'après l'examen de la série précédente, on n'y trouve jamais de chorde, tandis que les somites ne sont apparus que dans un cas unique.

La sensibilité d'une même ponte de grenouille présente donc, depuis le stade blastula jeune, jusqu'au début de la gastrulation, une évolution tant d'ordre *qualitatif* que *quantitatif* vis-à-vis de la centrifugation.

TABLEAU 6

Résultats de la centrifugation 1945/2, 23 h. après fécondation

I. Embryons étudiés: 67.
Embryons anormaux: 28 (42%).
II. Anomalies du système axial primaire (% calculés sur le total de 28):
Plis ou dédoublements partiels au niveau du cerveau: 14 (50%).
Dilatation antérieure de la chorde dorsale: 0.
Nodules chordaux dans le névraxe: 0.
Hypertrophie d'un otocyste primaire: 8 (28%).
III. Systèmes indépendants de l'axe primaire:
Total: 23 (82% calculé sur le total de 28).
contenant (sur le total de 28):
du névraxe (rhombencéphale et moelle): 9 (33%).
de la chorde: 0.
des somites: 3 (10%).
du pronéphros: 0.
des otocystes: 16 (57%).
des dérivés de crête neurale: 17 (61%).
de la glande adhésive: 2 (7%).

2. *Comparaison de trois expériences*

Après avoir ainsi analysé les résultats d'une expérience type, nous procéderons ensuite à une comparaison des résultats obtenus dans trois expériences semblables faites sur des pontes différentes.

Ces résultats sont exprimés sur les courbes des fig. 1 et 2 dans le texte: courbes A pour l'expérience 1945/2 qui vient d'être détaillée plus haut, courbes B et C pour deux autres expériences (1945/6 et 1941/1). Sur la fig. 1 nous représentons, en fonction du stade (exprimé en abscisses, en heures de développement à 20°) le pourcentage total des anomalies (trait plein), celui des systèmes axiaux autonomes (trait interrompu), celui des inclusions chordales dans le névraxe primaire (point et tiret), celui des dilatations antérieures de la chorde primaire (pointillés).

Sur la fig. 2 nous représentons, en fonction des mêmes abscisses, les pourcentages d'organes formés (rapportés au total des embryons *anormaux*): trait plein: névraxe; tirets: chorde; points et tirets: somites; pointillé: pronéphros.

Nous voyons ainsi que ces trois expériences montrent une évolution générale semblable, avec néanmoins des variantes suivant les pontes. Il est possible ainsi de dégager les conclusions suivantes:

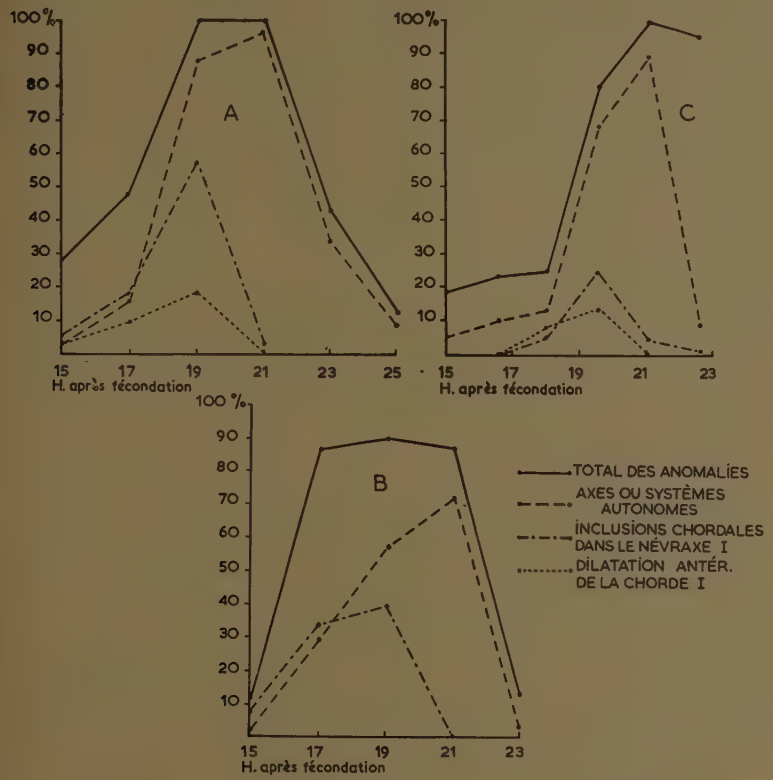


FIG. 1. Graphiques montrant la fréquence des anomalies (ordonnées) obtenues par la centrifugation des œufs à divers stades du développement (en abscisses: heures après fécondation, à 20°). Les courbes *A*, *B*, *C* se rapportent à trois expériences différentes. Trait plein: total des anomalies. Tirets: systèmes autonomes. Points et tirets: inclusions chordales dans le névraxe. Pointillés: dilatation antérieure de la chorde primaire.

(*a*) L'ectoblaste n'est sensible que pendant une phase limitée du développement: cette phase s'étend depuis la blastula moyenne jusqu'au début de la gastrulation.

(*b*) D'une manière générale, on peut distinguer deux types de réactions, bien que celles-ci soient réunies par des intermédiaires: α: la *sensibilisation subliminale*; son action se manifeste toujours en corrélation étroite avec le névraxe primaire et consiste en une hyperproduction de tissu neural, avec inclusion éventuelle de nodules chordaux au sein du tissu nerveux; rappelons que dans le

premier mémoire de cette série nous avons démontré que la production de ces anomalies nécessite une action cumulative de la sensibilisation et de l'influx inducteur normal; β: l'*activation autonome*, c'est-à-dire la formation d'organes ou de systèmes organiques en position hétérotopique, indépendamment du système primaire et qui, comme nous l'avons démontré, se manifeste directement, indépendamment du champ d'organisation primaire.

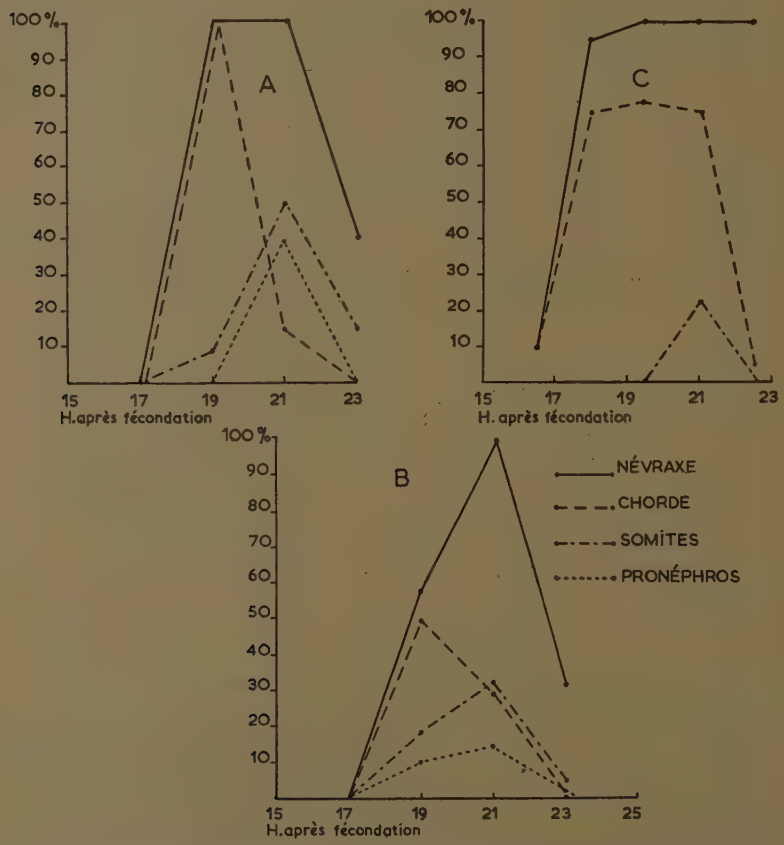


FIG. 2. Graphiques représentant, pour les trois mêmes expériences que celles de la fig. 1, la fréquence (en ordonnées) des organes secondaires obtenus après centrifugation à des stades croissants (abscisses: heures après fécondation, à 20°). Trait plein: névraxe; tirets: chorde; points et tirets: somites; pointillés: pronéphros.

Ce second mode de réaction semble répondre à des conditions plus étroitement définies que le premier. La phase du développement pendant laquelle l'activation autonome peut être suscitée est plus limitée dans le temps et leur fréquence présente un maximum très net correspondant au début de la gastrula-

tion (cf. fig. 1). Sur cette dernière figure, nous verrons d'ailleurs que la courbe de fréquence des activations autonomes a un tracé beaucoup plus étroit que celui de la courbe totale des anomalies, c'est-à-dire que: (1) aux stades jeunes, l'ectoblaste réagit uniquement de façon subliminale; (2) quand le germe est centrifugé au stade jeune gastrula (ou immédiatement avant), au moment où la sensibilité est maximale, les deux modalités de réaction sont associées.

Néanmoins, si nous comparons les résultats obtenus pour les diverses pontes, nous verrons que ces courbes ne sont pas nécessairement parallèles. Leur parallélisme n'est acceptable que dans l'expérience 1945/C2 (courbes *A*), mais ne l'est plus du tout dans les deux autres cas. Dans l'expérience 1945/C6 (courbes *B*) le surplus de sensibilisation subliminale est surtout net pour les stades initiaux, tandis qu'il apparaît surtout dans les stades terminaux dans l'expérience 1941/C1 (courbes *C*).

(*c*) *Au stade blastula, avec un maximum net pour la 19e heure, la sensibilisation subliminale s'accompagne de la formation de noyaux diffus de chordalisation dans le névraxe.* Ces chordalisations éparses sont tout à fait caractéristiques de cette période du développement et ne s'observent plus lorsque l'embryon a été centrifugé au stade jeune gastrula, bien qu'à d'autres points de vue, et notamment celui de la fréquence des axes secondaires, la sensibilité de l'ectoblaste soit à présent maximale.

(*d*) L'ensemble des expériences permet de conclure en toute netteté que les organes résultant de l'activation autonome de l'ectoblaste *ont toujours un caractère chordencéphalique ou tronco-caudal* (les deux étant souvent associés), *et jamais acrencéphalique.* Une légère réserve s'impose en faveur de la glande adhésive, dont la manifestation hétérotopique est fréquente; mais on sait qu'il s'agit là d'une réaction très 'facile' de l'ectoblaste des Anoures, et que l'on obtient même en explantation dans un milieu normal.

(*e*) *La nature des systèmes organiques autonomes varie d'après le stade auquel l'œuf a été centrifugé.* Il est aisé de comprendre qu'aux deux extrémités de leur courbe de fréquence (lorsque l'œuf a été centrifugé soit à la blastula jeune, soit à la gastrula quelque peu avancée) les systèmes autonomes aient un caractère 'mineur' et qu'ils se manifestent ainsi par de petits complexes neuroïdes, accompagnés d'otocystes, de ganglions et de petites nageoires dorsales: il s'agit de réactions que l'on peut qualifier de 'subneurales'.

Mais les vrais axes secondaires se présentent sous un jour plus inattendu. Ils contiennent *toujours* un névraxe, habituellement rhombencéphalique en avant, spinal en arrière. Il arrive que le système secondaire soit constitué par *ce seul névraxe*, tout au plus flanqué d'otocystes, dont la disposition n'est nécessairement ni normale, ni paire. Aussi remarquerons-nous que les courbes en trait plein de la fig. 2 enveloppent toujours les autres, la fréquence des réactions neurales dépassant constamment celle des réactions chordomésoblastiques.

Ces dernières présentent des variations notables en fonction du stade centrifugé. Dans l'expérience de la fig. 1, A, on constate que la réaction chordogène

est générale pour la 19e heure, tandis qu'elle tombe à un taux de 15 pour cent à la centrifugation de la 21e heure. Ce maximum est d'ailleurs moins élevé que pour celui de la chorde, n'atteignant que 50 pour cent pour les somites et 40 pour cent pour le pronéphros.

Dans l'expérience B (toujours fig. 2 dans le texte), il s'agit manifestement d'une ponte moins sensible, les fréquences de toutes les réactions étant plus faibles, mais on y retrouve une succession de phases à prépondérance chordale, somitique et pronéphritique dans les réactions. Cependant la courbe de fréquence chordale présente une chute moins brusque que dans le cas précédent.

Une situation un peu différente apparaît dans l'expérience C. Les réactions somitiques sont limitées et n'apparaissent qu'à un taux de 23 pour cent seulement et uniquement dans le lot de la 21e heure; et pas un seul pronéphros surnuméraire ne s'est manifesté au cours de cette expérience. Cependant la fréquence totale des axes surnuméraires reste élevée et sensiblement constante de la 18e à la 21e heure. Les résultats de cette expérience consistent donc surtout en des axes secondaires se composant uniquement de névraxe et de chorde suivant la modalité représentée sur la Planche 1, F. Contrastant avec cette figure nous verrons sur la Planche 2, D l'aspect d'un axe secondaire constitué uniquement de névraxe et de myotomes et provenant du lot de la 21e heure de l'exp. A (1945/2).

Il existe donc des différences individuelles dans la sensibilité des différentes pontes. Mais ces variantes ne sont que des modulations sur un thème commun qui peut se résumer comme suit: *la fréquence relative des organes constituant les axes autonomes s'établit suivant la progression: pronéphros, somites, chorde, névraxe; cependant les maxima de fréquence de la chorde d'une part, des somites et pronéphros d'autre part, correspondent à des centrifugations faites à des moments différents, plus précoces pour les chordes, plus tardives pour les somites et le pronéphros.*

(*f*) Enfin, signalons que dans les deux séries expérimentales 1945/C2 et 1945/C1 (A et C, fig. 1 dans le texte), il est apparu des signes discrets, mais indubitables, d'une action sur le chordomésoblaste primaire de l'embryon sous forme d'une hypertrophie de la partie antérieure de la chorde. Il s'agit visiblement d'une *chordalisation de la plaque préchordale*. Nous reviendrons plus loin sur cette modalité nouvelle d'anomalie du développement, car elle présente un intérêt suffisant pour qu'un chapitre entier lui soit consacré. Bornons-nous à signaler qu'elle est caractéristique de la centrifugation au stade blastula, avec un maximum *à la 19e heure. Cette période apparaît donc comme un stade où la centrifugation produit une chordalisation par trois mécanismes différents*: l'apparition de nodules chordaux dans le névraxe primaire, la prédominance du tissu chordal dans les axes secondaires, enfin une tendance à la chordalisation de la plaque préchordale. On peut penser qu'il ne s'agit pas d'une simple coïncidence et que ces trois manifestations différentes relèvent d'un mécanisme commun.

RÉSULTATS. 2. EFFETS COMPARATIFS DE LA CENTRIFUGATION AUX DIVERS STADES CHEZ D'AUTRES ESPÈCES

Ces résultats étant obtenus chez la Grenouille, il y avait lieu de se demander s'ils étaient strictement généralisables, ou si des modalités ou des variantes différentes ne se retrouveraient pas chez d'autres Amphibiens. Aussi avons-nous étendu nos recherches au plus grand nombre d'espèces possible, tant Urodèles qu'Anoures. Nous n'avons pas cependant toujours disposé d'un matériel aussi nombreux que chez *Rana temporaria*.

(*a*) En particulier, chez *Xenopus laevis*, faute de pontes suffisantes, l'étude systématique des effets de la centrifugation n'a pu être réalisée sur une aussi grande échelle que chez la Grenouille rousse. Néanmoins, les résultats obtenus sont suffisants pour dégager une impression d'ensemble.

Les œufs cultivés à 22° ont pu être centrifugés, d'heure en heure, depuis la 7e heure jusqu'à la 11e après fécondation, ce qui correspond aux stades allant de la morula moyenne à la gastrula jeune.

Les résultats obtenus sont très semblables à ceux de la Grenouille, avec toutefois certaines différences de détail. Le stade de la morula jeune, contrairement aux constatations faites chez la Grenouille, se montre déjà sensible; le maximum d'effet est obtenu dans la blastula moyenne, tandis qu'au stade gastrula, les réactions sont déjà diminuées et limitées à des systèmes mineurs, représentés surtout par des queues contenant du muscle et du mésenchyme.

Les différences qualitatives des résultats obtenus de stade en stade rappellent l'évolution que nous avons étudiée chez la Grenouille, mais les différences observées de stade en stade sont toutefois moins tranchées. Au stade le plus jeune, celui de la morula moyenne (7 h. de développement) le résultat obtenu est analogue à celui de la jeune blastula chez la Grenouille: c'est-à-dire des dédoublements partiels du névraxe primaire, et la présence éventuelle de nodules chordaux dans ce névraxe; ces nodules sont toutefois plus rares et ne dépassent pas une fréquence de 20 à 30 pour cent. A ces stades précoces, les systèmes autonomes sont encore rares et discrets (dérivés de crête neurale et otocystes).

Dès la 8ème heure (toute jeune blastula) et cela sans grand changement jusqu'à la 10e heure (blastula avancée), les systèmes autonomes sont fréquents et constituent de véritables axes secondaires. Mais c'est ici qu'intervient la différence majeure avec nos observations chez la Grenouille: il n'apparaît pas de différences systématiques entre les résultats obtenus à ces divers stades. Tandis que chez *Rana* nous observions une prépondérance chordale pour les stades jeunes, une prépondérance somitique pour les stades avancés, cette systématisation n'apparaît pas chez le Xénope. Quel que soit le stade de la blastula qui ait été centrifugé, on voit dans les divers axes secondaires d'un même lot, tantôt de la chorde, tantôt des somites, tantôt les deux sans que l'on puisse, dans la limite d'un matériel assez réduit, y constater des différences de fréquence significatives.[1])

[1] Il est toutefois possible, que disposant d'un matériel beaucoup plus abondant, nous aurions

Une dilatation de la chorde primaire, dans sa partie antérieure est apparue également au cours de ces expériences sur le Xénope et uniquement au stade de la blastula jeune, c'est-à-dire le même stade pour lequel le même résultat a été obtenu chez la Grenouille. Ici également cette action sur le chordomésoblaste primaire s'est limitée à un nombre assez réduit d'embryons (20 à 25 pour cent des cas). Nous reviendrons sur ces cas, particulièrement intéressants, dans le chapitre spécial qui leur est dévolu (cf. p. 142).

(*b*) Chez le Pleurodèle, la sensibilité de l'ectoblaste à l'égard de la centrifugation est plus faible que chez la Grenouille et le Xénope. Certaines pontes ne réagissent pas, tous les embryons restant normaux quel que soit le stade de la centrifugation. Dans les pontes qui paraissent sensibles, la période de sensibilité apparaît différente à la fois de celle de la Grenouille et du Xénope. Tandis qu'elle était plus précoce chez la seconde espèce que chez la première, elle apparaît plus tardive chez le Pleurodèle. Aucune réaction ne se manifeste après centrifugation de la blastula jeune, tandis que les stades gastrulas à fossette blastoporale déjà bien enfoncée sont encore sensibles.

Les anomalies produites par la centrifugation sont de deux ordres: des dédoublements partiels du névraxe primaire et la formation de systèmes 'autonomes', en dehors de ces axes. Comme chez la Grenouille, la première catégorie — qui traduit la sensibilisation subliminale de l'ectoblaste — se retrouve le long de toute la gamme des stades sensibles. Les systèmes autonomes au contraire dépendent d'une phase de sensibilité plus limitée, se bornant au début de la gastrulation. Les axes secondaires obtenus chez le Pleurodèle sont moins puissants (cf. mémoire I, Pasteels, 1953, fig. 6, p. 11), se bornant habituellement à des formations caudales contenant du muscle et des dérivés de crête neurale: ganglions, masses neuroïdes et mésenchyme. Ces queues accessoires exerçant une influence notable sur le mésoblaste ventral et même l'entoblaste primaire, nous aurons l'occasion de les étudier plus en détail dans le 3e mémoire de cette série.

Chez cette espèce, nous n'avons, en aucun cas, pu observer la formation de nodules chordaux dans le névraxe primaire, ni la chordalisation de la plaque préchordale. Notons d'ailleurs qu'il n'est pas apparu de chorde dans les petits axes secondaires.

(*c*) A cette sensibilité tardive du Pleurodèle, nous opposerons une sensibilité précoce chez un autre Urodèle: *Ambystoma punctatum*. Les anomalies obtenues sont identiques à celles du Pleurodèle, mais l'effet maximal apparaît dans la centrifugation du stade blastula (3 h. avant le début de la gastrulation à 17°). On obtient à ce stade de petites queues secondaires (muscles et crête neurale), ou parfois un rhombencéphale secondaire, accompagné d'otocystes; dans une blastula plus jeune ou au tout début de la gastrulation, c'est également sous la forme de dédoublements partiels du névraxe que se manifeste la sensibilisation sub-

pu déceler, par une méthode statistique, de telles différences. Néanmoins elles seraient de toute façon moins accusées qu'elles ne l'étaient chez la Grenouille.

liminale. Pas plus que chez le Pleurodèle nous n'avons pu observer de chordalisations, sous aucune de ses modalités.

(*d*) Dans le premier des mémoires de cette série, nous avons déjà décrit la sensibilisation subliminale caractéristique de l'Axolotl et analysé son mécanisme. Rappelons qu'il s'agit chez cette espèce, de dédoublements partiels du prosencéphale et du rhombencéphale. La toile choroïdienne du 4e ventricule est souvent remplacée par une masse solide de tissu nerveux, à côté duquel se voient des amas de ganglions et de petits otocystes. Parfois ces formations neurales supplémentaires ont un caractère nettement hétérotopique: nous avons signalé et représenté des lobes et fossettes olfactives au niveau du toit du rhombencéphale, ainsi qu'un petit rhombencéphale accessoire se détachant de la moelle (cf. mémoire I, Pasteels, 1953, Planche 3, A et D).

TABLEAU 7

Nombre de dédoublements partiels du cerveau obtenus chez l'Axolotl dans une même ponte

Temps de développement	*Stade*	*Nombre d'embryons étudiés*	*Nombre d'anomalies*
27 h.	Blastulas jeunes	60	0
29 h.	Blastulas	36	16(±44%)
31 h.	Blastulas	38	22(±58%)
33 h.	Blastulas avancées	47	31(±69%)
35 h.	Toutes jeunes gastrulas	38	18(±47%)

Une telle description vaut pour tous les stades sensibles de l'Axolotl, stades qui s'échelonnent depuis la blastula jusqu'à la jeune gastrula. *La fréquence de ces anomalies peut varier*, comme le montre le Tableau 7 relatant les résultats de l'expérience la plus complète, *mais la nature des résultats est identique, quel que soit le stade étudié*. On n'observe jamais la formation de complexes organiques autonomes, ni de chordalisation du névraxe. Toutefois sur l'ensemble de plusieurs centaines d'embryons examinés, dans deux cas nous avons obtenu une chordalisation partielle de la plaque préchordale.

(*e*) Une étape de plus dans cette décroissance de la sensibilité des pontes, et nous arrivons à la situation observée chez le Discoglosse. Ici nous nous sommes ingénié à multiplier les centrifugations à des accélérations variées, pendant des temps divers, et à tous les stades. Le plus souvent le résultat était nul. Parfois cependant, chez quelques embryons, nous avons obtenu quelques malformations locales du cerveau primaire.

(*f*) Chez les trois Tritons étudiés (*T. vulgaris, helveticus,* et *alpestris*), la centrifugation de la blastula et de la gastrula s'est toujours avérée inefficace.

En résumé: (1) Aux stades de la blastula et de la jeune gastrula, il apparaît entre les œufs des diverses espèces des différences considérables de sensibilité à l'égard de la centrifugation; cette sensibilité est très forte chez la Grenouille

rousse et chez le Xénope, plus faible chez le Pleurodèle et l'*Ambystoma punctatum*, réduite chez l'Axolotl, à peu près nulle chez le Discoglosse, et nulle chez les Tritons.

(2) La période de sensibilité maximale varie également chez les diverses espèces. Elle est précoce chez le Xénope (blastula moyenne), plus tardive chez l'*Ambystoma* (blastula avancée), plus tardive encore chez le Pleurodèle (jeune gastrula).

(3) Les différences qualitatives obtenues aux divers stades par une même centrifugation s'atténuent au fur et à mesure que décroissent les sensibilités spécifiques. Particulièrement marquées chez la Grenouille, elles deviennent nulles chez l'Axolotl.

(4) La formation de noyaux chordaux dans le névraxe primaire, si caractéristique des centrifugations au stade blastula jeune chez la Grenouille ne s'est retrouvée que chez le Xénope, mais pas chez les autres espèces.

(5) Il en est de même pour la chordalisation de la plaque préchordale, sauf des cas très sporadiques observés chez l'Axolotl.

RÉSULTATS. 3. ETUDE COMPARATIVE DES VARIATIONS DE DURÉE DE CENTRIFUGATION CHEZ *RANA TEMPORARIA*

Comme nous l'avons vu, lorsqu'on centrifuge une série de lots de la même ponte, à des stades croissants, on observe une variation du nombre d'œufs qui réagissent. Mais, de plus, on constate que la *nature* des réponses varie d'après le stade centrifugé.

On pourrait se demander si ces deux expressions, *quantitative* et *qualitative* de la sensibilité, ne seraient pas liées: des œufs amenés à réagir plus, seraient entraînés nécessairement à réagir *différemment*. On peut aussi concevoir que si le nombre de réactions et leur qualité dépendent toutes deux du stade de la centrifugation, il n'existe néanmoins pas de lien direct entre ces deux modalités de la réponse.

A première vue, il est facile de départager ces deux éventualités par une expérience qui consisterait à prendre des lots d'une même ponte et d'un même stade et de les centrifuger avec des intensités croissantes. En réalité nous avons éprouvé quelques difficultés à réaliser cette expérience du fait que l'effet maximal produit à un stade déterminé est très rapidement atteint par l'accélération de 460 g. qui représentait la vitesse minimale de l'instrument dont nous disposions. Nous nous sommes donc efforcé de réduire le temps de centrifugation à cette vitesse donnée.[1]

[1] Il est évidemment difficile d'obtenir une centrifugation à une accélération définie pendant un temps court. En pratique, nous procédions de la façon suivante: la centrifugeuse était déclenchée au premier plot lequel, après établissement du régime normal aurait dû donner une accélération de 460 g. Ensuite après un temps déterminé, nous coupions le contact. Il y a lieu donc d'ajouter au temps de centrifugation théorique le temps supplémentaire dû à l'inertie de l'appareil; mais par ailleurs, il est clair que si le contact était établi pendant 10 à 30 secondes, le plein régime de l'appareil était loin d'être établi.

(1) Dans une première expérience nous avons pris des œufs au stade très sensible de la jeune gastrula et comparé les effets d'une centrifugation de 50 et de 10 secondes (pour la durée de 50 secondes, l'effet maximal était déjà obtenu). La différence quantitative des résultats est patente. Les deux lots comprenaient 55 embryons. Pour 50 secondes de centrifugation, tous les embryons du lot ont réagi sans exception en formant de volumineux axes secondaires contenant du névraxe et souvent des somites, de la chorde. Quant au pronéphros, il était particulièrement abondant dans cette expérience. Il s'agit toutefois de pronéphros formé par le mésoblaste ventral (et non par l'ectoblaste activé) dont le potentiel morphogénétique avait été élevé au contact de l'axe secondaire (suivant une modalité qui sera précisée dans le 3e mémoire de cette série).

Dans le lot centrifugé pendant 10 secondes seulement, 31 des 55 embryons ont formé des axes secondaires, en général moins volumineux. Ils sont toutefois de même nature que dans le lot de 50 secondes, et la fréquence des divers tissus formés par l'ectoblaste activé est la même, du moins si l'on se rapporte au total des embryons ayant réagi (et non au total des embryons traités). Ceci se verra aisément sur le Tableau 8 où l'on notera comme seule différence appréciable la diminution du nombre de pronéphros 'induits' à partir du mésoblaste ventral, et il est compréhensible de voir les chances de cette induction diminuer lorsque les axes secondaires sont moins volumineux.

TABLEAU 8

Deux lots de jeunes gastrulas de la même ponte centrifugés pendant 50 sec. et 10 sec.

	Total	*Fréquence des axes secondaires*	*Nature des organes (% calculés d'après le nombre d'axes secondaires)*					
			Névraxe	*Otocystes*	*Chorde*	*Somites*	*Crête neurale*	*Pronéphros*
50 sec.	55	55	53 (96%)	45 (82%)	15 (27%)	15 (27%)	29 (53%)	23 (42%)
10 sec.	55	31	27 (87%)	26 (84%)	8 (26%)	11 (35%)	20 (64%)	5 (16%)

(2) Une seconde expérience envisage le cas de blastulas jeunes. Le maximum de réactions est obtenu par une centrifugation de 2 minutes à 460 g. et se traduit alors par une fréquence de 65 pour cent d'anomalies, consistant en des dédoublements partiels du névraxe primaire et quelques systèmes autonomes mineurs, sous forme d'otocystes et de dérivés de crête neurale. Il s'agit effectivement d'un effet maximal qui ne peut être dépassé à ce stade, le même résultat étant obtenu indifféremment si l'on augmente l'accélération ou la durée de la centrifugation.

Si l'on diminue cette durée, la fréquence des anomalies baisse progressivement. Pour 30 secondes de centrifugation, par exemple, les réactions se limitent à 10 pour cent du lot centrifugé. Avec un nombre aussi réduit de résultats il devient difficile d'établir une évaluation numérique satisfaisante. Néanmoins l'examen des divers lots ne montre pas de différences qualitatives.

(3) *La comparaison des résultats de ces deux expériences* montrera enfin à quel point la *qualité* du résultat obtenu est uniquement fonction du *stade* qui a été centrifugé et non de l'*intensité* de la réaction produite. En effet, les jeunes gastrulas centrifugées pendant 10 secondes et les jeunes blastulas centrifugées pendant 2 minutes montrent une fréquence égale d'anomalies et cependant la qualité du résultat est entièrement différente dans les deux cas.

Conclusion: *les différences qualitatives des transformations de l'ectoblaste ne sont pas fonction de l'intensité (durée ou accélération) de la centrifugation, mais dépendent uniquement du stade où l'œuf a été centrifugé.*

RÉSULTATS. 4. ACTION DE LA CENTRIFUGATION SUR LE CHORDO-MÉSOBLASTE PRIMAIRE: LA CHORDALISATION DE LA PLAQUE PRÉCHORDALE

Si les effets de la centrifugation de la blastula et de la jeune gastrula se manifestent par une activation de l'ectoblaste, il n'en est pas moins vrai que dans certaines conditions le chordomésoblaste puisse être également affecté.

Chez la Grenouille et le Xénope, nous avons vu en effet qu'un certain nombre d'embryons centrifugés au stade blastula jeune montraient une curieuse anomalie se traduisant par une hypertrophie de la partie antérieure de la chorde. Le même résultat a été retrouvé, mais très sporadiquement, chez l'Axolotl. Une telle transformation du chordomésoblaste doit nécessairement modifier son pouvoir inducteur et c'est ce point que nous voudrions illustrer de quelques exemples.

Voici un premier cas, obtenu chez la Grenouille (Planche 3, A, B, C). Comme on le voit sur la 1ère figure (A) la tête est compacte, avec un pharynx petit. Dès les premières coupes de l'embryon apparaît une chorde à contours irréguliers. Il n'y a pas de prosencéphale mais une masse irrégulière de tissu nerveux, sans cavité ventriculaire. Lobes olfactifs et yeux sont totalement absents. Plus en arrière (Planche 3, B) on aperçoit à côté de la chorde principale, des noyaux de chordalisation diffus se manifestant dans le mésenchyme céphalique, noyaux qui se réunissent à la chorde principale, de sorte que nous voyons bientôt un amas considérable de chorde, à contours très irréguliers. Le 'cerveau' est toujours remplacé par une masse compacte, irrégulière, sans ventricules. Dans la région nucale (fig. C) le pharynx se dilate, il y apparaît des poches branchiales minces, la chorde est encore fortement dilatée, le rhombencéphale est compact, sans ventricule et sans toile choroïdienne dorsale. La dilatation chordale se voit encore au niveau des premiers somites, mais le névraxe prend de plus en plus son aspect habituel. Le tronc est normal.

Nous emprunterons à nos expériences sur le Xénope un cas plus mitigé (Planche 3, D, E, F). Ici aussi lobes et fosses olfactives sont absents. Dès les premières coupes de l'embryon (fig. D) on voit sous un diencéphale petit, mais à cavité ventriculaire bien développée un œil cyclope. La partie antérieure de la chorde apparaît déjà au niveau de la partie postérieure de cet œil (fig. E). Plus en

arrière encore, au niveau des premiers somites, la dilatation de la chorde est considérable, tandis que le rhombencéphale est manifestement déficient, ses plaques alaires étant trop minces et la lame basale trop élargie (fig. F).

Conclusion: au stade blastula jeune, la centrifugation peut entraîner *une chordalisation de la plaque préchordale; cette transformation entraîne une déficience quantitative et qualitative de l'induction du cerveau antérieur*. La valeur très particulière de ce type de microcéphalie sera envisagée dans la discussion générale (dernière partie de cette série).

CONCLUSIONS ET RÉSUMÉ

(La discussion générale sera reportée à la fin du III^e mémoire)

1. Une étude comparée de la sensibilité à la centrifugation d'œufs de *Rana temporaria* provenant d'une même ponte, prélevés à des stades successifs montre que des anomalies ne peuvent être obtenues que pendant une phase assez courte du développement. Cette phase s'étend de la 17^e à la 25^e heure du développement (à 20°), c'est-à-dire depuis la blastula moyenne jusqu'à la gastrula au stade du blastopore en petite faucille. Le maximum de réaction apparaît vers la 21^e heure (toute jeune gastrula).

2. Différentes pontes de *Rana temporaria* présentent des différences parfois marquées de sensibilité, tant en ce qui concerne le pourcentage général des anomalies obtenues que dans les modalités de ces réactions, mais ceci dans une moindre mesure.

3. Néanmoins, de la comparaison des résultats obtenus dans diverses pontes de *Rana temporaria*, certaines conclusions générales peuvent être dégagées:

(*a*) Deux types de réactions peuvent être distinguées: (i) des vésicules neurales supplémentaires, ou des noyaux chordaux inclus dans le névraxe, manifestations dues à une action cumulative d'une 'sensibilisation subliminale' et de l'influx inducteur normal; (ii) des axes embryonnaires, incomplets ou mal équilibrés, en position ectopique et produits par une 'activation autonome' de l'ectoblaste. La seconde réaction intervient dans des limites de temps beaucoup plus précises que la première.

(*b*) En ce qui concerne les effets de la sensibilisation subliminale, il y a lieu de noter que les noyaux chordaux inclus dans le névraxe ne se montrent qu'après centrifugations précoces (fin de la blastulation), tandis que les vésicules neurales supplémentaires se montrent autant dans les centrifugations tardives que précoces.

(*c*) Les axes secondaires issus d'une activation autonome contiennent toujours un névraxe, associé souvent mais pas nécessairement avec du chordomésoblaste. La fréquence de ces organes mésoblastiques varie de façon absolue, et de façon relative suivant les stades. De façon absolue: la chorde est plus fréquente que les somites, ceux-ci plus fréquents que les tubes pronéphritiques. De façon relative: les maxima de fréquence de la chorde, des

somites, du pronéphros se succèdent suivant l'ordre chronologique des centrifugations.

4. Les résultats obtenus chez *Xenopus laevis* sont très semblables à ceux obtenus chez la Grenouille, toutefois la période de sensibilité est plus précoce.

5. Chez le Pleurodèle, l'ectoblaste paraît moins sensible à la centrifugation. Les axes secondaires sont exclusivement de type caudal. Ici, la période de sensibilité est plus tardive.

6. Chez *Ambystoma punctatum*, les réactions sont semblables à celles du Pleurodèle, mais correspondent à une période de sensibilité précoce (blastula).

7. Chez l'Axolotl, la sensibilité s'étend de la blastula jeune à la jeune gastrula, sans aucune différence quantitative entre ces divers stades. Le nombre d'anomalies est toujours limité à une partie du lot traité et ne consiste qu'en vésicules neurales supplémentaires. On y trouve donc une des manifestations de la sensibilisation subliminale, à l'exclusion de l'autre: les inclusions chordales.

8. Les œufs du Discoglosse ne présentent qu'une sensibilité très limitée, et ceux des trois Tritons (*T. vulgaris, helveticus,* et *alpestris*) une sensibilité nulle aux effets de la centrifugation.

9. Les différences qualitatives éventuelles des réactions ne dépendent pas de l'intensité (durée ou accélération) de la centrifugation, mais uniquement du stade où l'œuf a été centrifugé.

10. Chez la Grenouille et le Xénope, on observe dans un certain nombre de cas, et uniquement après des centrifugations précoces (blastula), une anomalie inédite se caractérisant par la chordalisation de la plaque préchordale, et corrélativement une déficience en quantité et en qualité de l'induction cérébrale.

11. Chez la Grenouille, la centrifugation de la blastula — et ceci est spécifique de ce stade — entraîne la formation de chorde en excès par trois mécanismes différents: la chordalisation de la plaque préchordale, la naissance de nodules chordaux inclus dans le névraxe, et une prépondérance chordale dans les axes secondaires.

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(Une bibliographie plus étendue suivra la fin de la III[e] partie.)

EXPLICATION DES PLANCHES

PLANCHE 1

A–C. Résultats de la centrifugation de *Rana temporaria*, 15 h. après la fécondation, à 20° (blastula jeune). Vésicules cérébrales supplémentaires et noyaux chordaux inclus dans le névraxe. La figure A représente une coupe passant par le prosencéphale d'un embryon, les fig. B et C des coupes successives par la région rhombencéphalique d'un autre embryon. *Cav. cer. II*, cavité cérébrale secondaire; *Ch. dil.*, chorde dilatée; *Ch. incl.*, inclusions chordales; *Ot.*, otocystes; *Rhomb. II*, rhombencéphale secondaire; *Stom.*, stomodaeum.

D, E. Résultat de la centrifugation d'un embryon de *Rana temporaria*, 17 h. après la fécondation, à 20° (blastula moyenne). Coupes successives par la tête d'un même embryon. *Cav. dig. II*, cavité digestive secondaire; *Cerv.*, cerveau; *Ch.*, chorde; *Ot.*, otocyste.

F. Résultat de la centrifugation d'un embryon de *Rana temporaria*, 19 h. après la fécondation, à 20° (blastula avancée). Complexe organique comprenant des vésicules rhombencéphaliques, de la chorde et du mésenchyme primaire.

Planche 2

A–C. Autre cas de centrifugation de *Rana temporaria*, 19 h. après la fécondation, 20° (blastula avancée). En B, le détail de la coupe A; en C, la même région, mais un peu plus en arrière. Tissu de transition, chordo-neural, se rattachant au rhombencéphale.

D. Résultat d'une centrifugation d'un embryon de *Rana temporaria*, 21 h. après la fécondation, à 20° (toute jeune gastrula). Axe secondaire dans la région ventrale de l'embryon. *Ch. II*, chorde secondaire; *Med. II*, moelle secondaire; *Pron. II*, pronéphros secondaire; *Som. II*, somites secondaires.

Planche 3

Chordalisation de la plaque préchordale. Les figures A, B, C d'une part et D, E, F de l'autre donnent des coupes successives de deux embryons, le premier de *Rana*, le second de *Xenopus*.

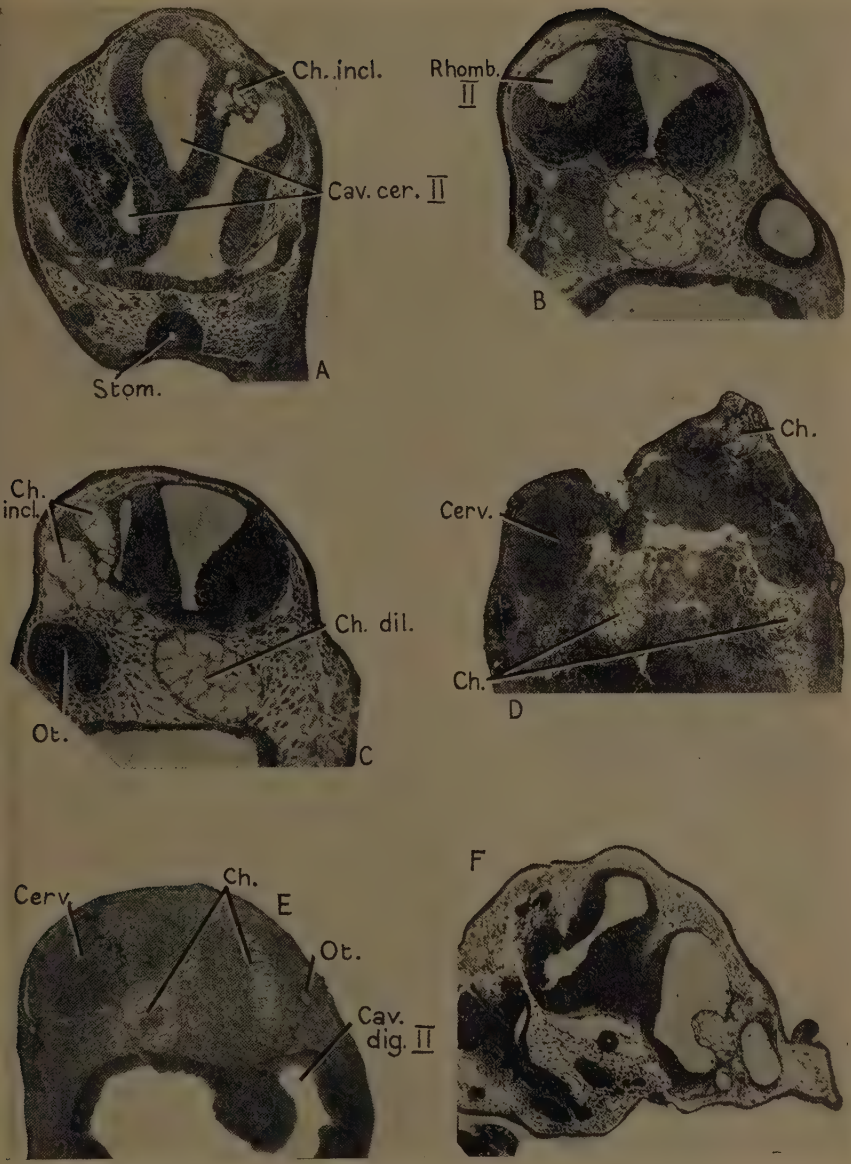

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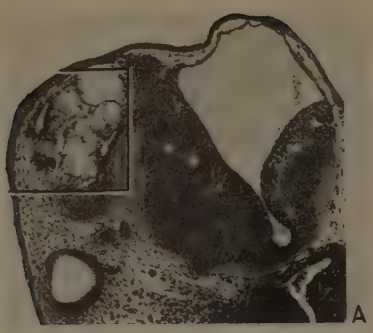
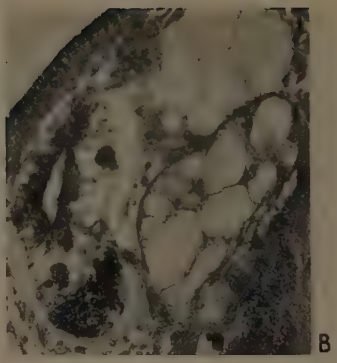
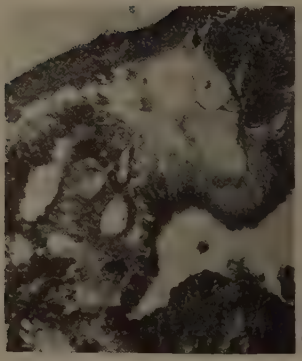
Planche I







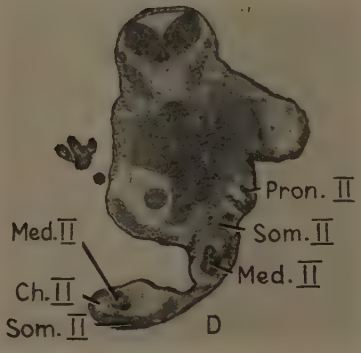


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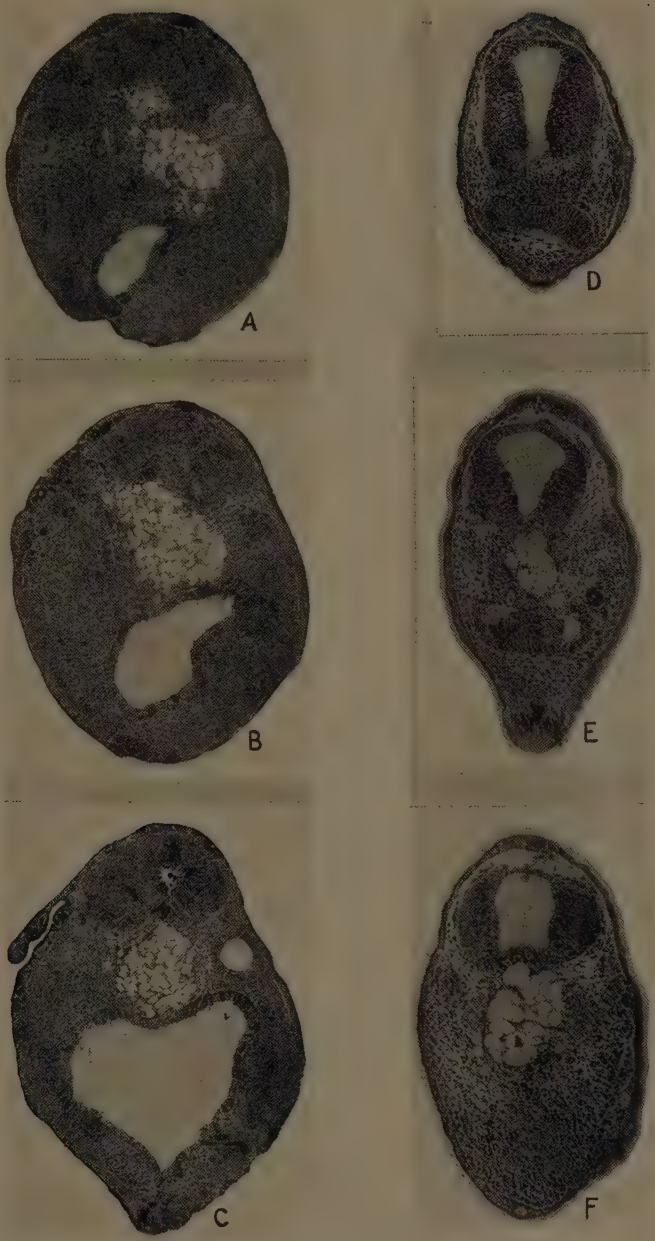
Planche 2



J. PASTEELS
Planche 3

The Yolk-sacs of *Erinaceus europea* and *Putorius furo*

by B. MORRIS[1]

From the Department of Zoology, University College of North Wales, Bangor

WITH ONE PLATE

INTRODUCTION

SINCE in the rabbit maternal plasma proteins are found in the yolk-sac cavity, and some at least pass into the foetal circulation by way of the vitelline vessels (Brambell *et al.*, 1952), a comparison with two such widely different forms as the hedgehog and ferret was thought to be of interest. The development and structure of the yolk-sacs, and the nature of their contents, in *Erinaceus europea* and *Putorius furo* are therefore described below.

MATERIAL AND METHODS

The hedgehog material was collected in Caernarvonshire during the summers of 1949 and 1950. The first pregnancies were obtained in early May. Some of the hedgehogs and ferrets were killed by ethyl chloride anaesthesia, others by intravenous magnesium chloride. The ferrets were killed at stages of pregnancy between 16 and 21 days; the duration of pregnancy was timed from the commencement of copulation.

Ligatures were applied to the uterus at either side of each uterine swelling, and the swellings were then removed. Some were fixed in aqueous Bouin's fluid and preserved in 70 per cent. alcohol. These were later sectioned and stained in Ehrlich's haematoxylin and 1 per cent. aqueous eosin. The remainder were rapidly frozen in a tube containing isopentane cooled in a Thermos flask containing a mixture of 70 per cent. alcohol and solid carbon dioxide. The yolk-sac fluid was later dissected out as a globule of ice, which was then allowed to thaw (Brambell *et al.*, 1949). The fluid was centrifuged and transferred to a tube for weighing. The amount of yolk-sac fluid obtained from some swellings was small, and in such cases the fluid from a number of swellings in the same uterus was pooled.

A small quantity of human thrombin solution was added to some of the yolk-sac fluid samples to determine the presence or absence of fibrinogen. The remainder of the yolk-sac fluid was used to determine the concentration of protein

[1] *Author's present address*: Zoology Department, University Park, Nottingham, England.

[J. Embryol. exp. Morph. Vol. I, Part 2, pp. 147–60, June 1953.]

nitrogen. The method used was the simplified ultra-micro Kjeldahl technique described by Shaw & Beadle (1949). The protein nitrogen of the maternal serum was determined by micro Kjeldahl for comparison.

THE YOLK-SAC OF *ERINACEUS EUROPEA*

The placentation of *Erinaceus europea* has been described by Hubrecht (1889). Implantation is interstitial and antimesometrial.

The earliest stages examined were those of blastocysts which had already commenced implantation, the yolk-sac cavity of the smallest measuring 0·18 mm. across its greatest diameter. At this stage the decidua capsularis is being formed, but has not yet completely separated the implanting blastocyst from the uterine lumen.

The trophoblast of the blastocyst has already begun to extend into the surrounding maternal tissue, and is thickest at the antimesometrial pole where the embryonic plate will differentiate. Elsewhere it is two or three cells thick. Hubrecht described the appearance of lacunae within the trophoblast of the lateral walls of the blastocyst, and their subsequent formation in the mesometrial and antimesometrial walls. He attributed their formation to the very rapid enlargement of the blastocyst outpacing the proliferation of the trophoblast cells. In the earliest stages examined these lacunae, in which maternal blood is present, do not usually appear entirely within the trophoblast, but are formed, at first, between it and the adjacent decidua.

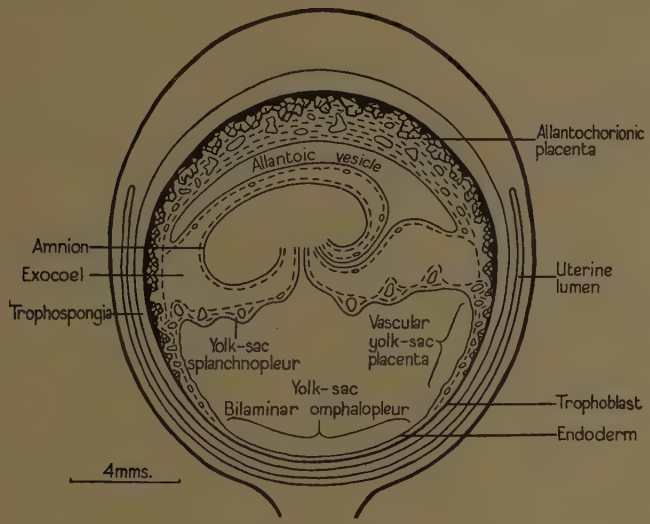
The blood-vessels in the decidual tissue adjacent to the blastocyst have increased in size and number, presumably to permit of an increased flow of blood to this region. They are lined by a distinct endothelium, the cells of which protrude into the lumina of the vessels. These enlarging endothelial cells will eventually form the *trophospongia*: a region of maternal decidual tissue closely adjacent to the blastocyst (Hubrecht).

The blastocyst enlarges rapidly, and when the formation of the embryonic knob at the antimesometrial pole is completed the diameter of the yolk-sac has increased to 0·46 mm. The yolk-sac of the latest embryonic plate stage available, in which mesoderm has not differentiated, measures 2·0 mm. across its greatest diameter.

After the formation of the mesoderm, and the appearance in it of the coelom, the amniotic folds grow up into the cavity between the embryonic plate and the antimesometrial trophoblast. The mesoderm extends mesometrially, between the endoderm and the trophoblast, converting a part of the bilaminar omphalopleur into a trilaminar structure. At first the exocoel is not extensive, being confined to the margin of the embryonic area, but soon it enlarges and extends mesometrially to separate the vascular splanchnic mesoderm (area vasculosa) from the avascular somatic mesoderm.

The vascular yolk-sac placenta. The mesoderm continues to extend mesometrially between the endoderm and the trophoblast, and when the vascular

yolk-sac placenta has attained its maximum development only the mesometrial third of the yolk-sac wall remains bilaminar. The trophoblast becomes converted into a spongy tissue in which numerous lacunae are formed. Maternal blood circulates in these lacunae which are continuous with the large blood-spaces which have been formed in the adjacent trophospongia. At certain scattered points some of the vessels of the area vasculosa, with their covering of mesenchyme, penetrate into the spongy trophoblast, and the omphaloidean villi which are thus formed bring the foetal blood into close proximity with the maternal blood present in the lacunae (Hubrecht).



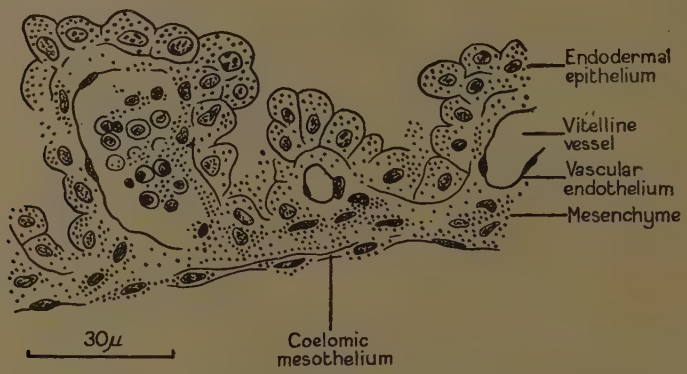
TEXT-FIG. 1. Diagram of a transverse section of the uterus of the hedgehog at about mid-pregnancy.

The Plate, fig. A, shows a portion of the vascular yolk-sac placenta at its greatest development. At this stage the maternal and foetal circulations are generally separated from one another by two tissues—the trophoblast which is usually only one cell thick, and the foetal endothelium of the vitelline vessels. Thus in the hedgehog the omphaloidean placenta is haemochorial in character, and the vascular relations of the omphaloidean and allantochorionic placentae are similar.

The larger omphaloidean villi are formed in the antimesometrial region of the trilaminar omphalopleur, and they penetrate into the spongy trophoblast almost as far as the trophospongia. The somatic mesoderm cannot be discerned as a separate layer, but later in development it becomes noticeable in those regions of the trilaminar omphalopleur where omphaloidean villi have not been developed, as a single layer of squamous cells.

Later some of the large vitelline vessels, with their covering of endoderm and mesenchyme, protrude into the cavity of the yolk-sac. By increasing the internal surface area of the yolk-sac splanchnopleur they may facilitate absorption from the fluid contents.

The rapidly enlarging embryo causes the yolk-sac splanchnopleur to become partially inverted and concurrently brings about the withdrawal of the more antimesometrially situated vitelline vessels and their surrounding mesenchyme from their sites in the spongy trophoblast (Text-fig. 1). The extension of the exocoel, resulting in the separation of the yolk-sac splanchnopleur from the chorion and the withdrawal of the vascular mesodermal cores of the omphaloidean villi, is accompanied by the expansion of the allantois. The allantoic meso-



TEXT-FIG. 2. A portion of the yolk-sac splanchnopleur of the hedgehog at a very late stage of pregnancy.

derm extends mesometrially, increasing the area of the allanto-chorion. In the hedgehog no anastomoses are formed between the allantoic and vitelline circulations, such as occur in the rabbit (Duval, 1892), although these are closely adjacent to one another at some stages.

As the embryo enlarges the yolk-sac splanchnopleur is further invaginated and becomes thrown into folds, and its endodermal epithelium thickens progressively, the constituent cells becoming, at first, cuboidal and later low columnar. Their apical ends are rounded and their flat basal ends rest on a distinct basement membrane (Text-fig. 2). The mesenchyme which was previously of a loose texture, is now far more compact. In the latest stage available the yolk-sac appears, in transverse sections, as a slit-like crescentic cavity, and the much folded yolk-sac splanchnopleur has attained its greatest degree of differentiation.

The avascular yolk-sac placenta. Avascular yolk-sac placentation is initiated by the fusion of the trophoblast of the blastocyst with the uterine mucosa. In the early stages of pregnancy the entire yolk-sac wall was bilaminar and avascular,

but with the formation of the mesoderm and the extension of the area vasculosa, the avascular yolk-sac placenta diminishes in extent. In Text-fig. 1 the avascular yolk-sac placenta is restricted to a small area at the mesometrial pole. Hubrecht has shown that in the hedgehog the mesometrial extension of the mesoderm is never completed, so that a small bilaminar area persists mesometrially throughout pregnancy.

Due to the rapid enlargement of the embryo the tissues of the uterine wall are stretched. At mid-pregnancy the mesometrial uterine wall is about 2·0 mm. thick, whereas in late stages its thickness has been reduced to about 0·7 mm.

Mesometrially the endodermal cells are squamous, and this form is maintained until late pregnancy, when they thicken considerably. However, in the latest stages examined these cells have, apparently, also been affected by stretching, for they have returned to their earlier squamous form.

In these late stages the trophoblast and inner region of the trophospongia are indistinguishable from one another, and together they form a compact narrow band of tissue (Plate, fig. B). The numerous blood lacunae which were present in these tissues at earlier stages have now completely disappeared.

Reichert's membrane. In those regions of the trilaminar omphalopleur in which omphaloidean villi have not been developed, the somatic mesoderm can be distinguished from the splanchnic mesoderm even before the exocoel has extended to separate them. At first the somatic mesoderm is composed of squamous cells, but by mid-pregnancy these cells have thickened. At this stage a structureless hyaline layer makes its appearance between the somatic mesoderm and the trophoblast. Hubrecht briefly noted the time of its appearance and that it thickens in later stages and persists to term. It first becomes noticeable as a very thin layer, about 5μ in thickness, between the somatic mesoderm and trophoblast of the lateral walls of the conceptus, and is absent from the mesometrial wall of the yolk-sac until later. It increases in thickness as pregnancy proceeds and in the latest stage it is generally about 15μ thick at the mesometrial pole, but along the lateral walls of the conceptus it attains a thickness of up to 25–30μ (Plate, fig. B), and it extends up to the margin of the allantochorionic placenta. It is usually very closely adherent to the somatic mesoderm and trophoblast of the lateral walls and the endoderm and trophoblast of the mesometrial wall. However, at some points it has become detached from the underlying trophoblast. In the latest stages examined the somatic mesoderm of the lateral walls of the conceptus, like the endoderm of the mesometrial wall, has become stretched thin, and is again squamous.

THE YOLK-SAC OF *PUTORIUS FURO*

In the ferret implantation is of the central type, the blastocysts remaining free in the uterus for some considerable time. At 9 days the blastocyst is still surrounded by its zona pellucida (Hamilton, 1934), and the trophoblast commences to fuse with the uterine epithelium at about 14½ days (Strahl & Ballman, 1915).

By the 16th day the trophoblast is in contact with the uterine wall over almost the whole of its antimesometrial hemisphere, and blunt, finger-like trophoblastic villi have been formed which penetrate into the glandular mucosa. The greatest depth of penetration, 165μ, is attained in the region adjacent to the vascular splanchnic mesoderm. At the tips of the villi, and also along their sides, the epithelia of the uterine glands and the cells of the interglandular connective tissue are degenerating. The nuclei of the maternal cells thus affected are pycnotic and in various stages of disintegration.

Mesometrially the blastocyst is not in contact with the uterine epithelium, and in this region the uterine mucosa remains unchanged, being essentially similar in structure to the non-pregnant oestrus uterus. At the time of neural tube formation the area vasculosa has spread to cover the antimesometrial half of the blastocyst, and the trophoblast is generally two cells thick, except in the mesometrial region, where it is composed of a single layer of squamous cells.

The maternal tissues are not so highly vascular as in the hedgehog. The larger maternal blood-vessels pass radially in the connective tissue between the trophoblastic villi, and only at isolated points are they in close proximity to the trophoblast.

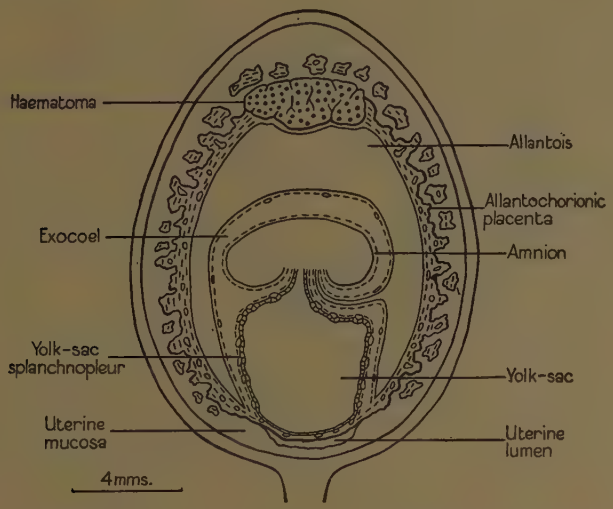
The structural changes which occur in the yolk-sac wall during the next 5 days of pregnancy have been described by Strahl & Ballman (1915). These changes are mainly associated with the increase in size of the trophoblastic villi and with the rapid extension of the allantois. The trophoblastic villi penetrate more deeply into the maternal tissue and become hollowed out. The cavities thus formed become partially filled with mesenchyme cells which are stellate and squamous in form and have been derived from the somatic mesoderm of the trilaminar omphalopleur (Plate, fig. C).

By the 18th day a large haematoma has been formed at the antimesometrial pole, and at 18½ days the mesoderm has completed its extension mesometrially and the yolk-sac placenta has attained its maximum development. The maternal and foetal blood circulations are separated from one another by the maternal endothelia, the trophoblastic ectoderm which is generally one cell thick, the mesenchyme, and the foetal endothelia. The trophoblastic villi have widened considerably, and at 19 days their mesenchymatous cores are still avascular, and they remain avascular until after the allantois has spread over them, when foetal blood-vessels derived from the vascular allantoic mesoderm can be discerned within them.

The volume of the yolk-sac increases up to about the 19th day, subsequently decreasing. At 21 days each yolk-sac yields about 0·16 ml. of fluid.

The arrangement of the embryonic membranes at 20 days is shown in Text-fig. 3. The allantois almost completely fills the exocoel, and the somatopleur and splanchnopleur are still in contact with one another at the mesometrial pole. By the 21st day the exocoel has extended to completely separate them, and the

vascular wall of the yolk-sac is neither in contact with the uterine wall at any point, nor with the contents of the uterine lumen, and omphaloidean placentation is terminated.



TEXT-FIG. 3. Diagram showing the morphological relations of the embryonic membranes of the ferret at 20 days.

Since the establishment of the omphaloidean placenta, which at the 16th day was mainly bilaminar, the yolk-sac membrane has undergone numerous structural changes. A thickening of the squamous endodermal epithelium is noticeable at 18- and 19-day stages, and by the 21st day the epithelium is composed throughout of cuboidal cells, the flattened basal ends of which rest on a basement membrane. The vitelline vessels are large, and are lined by a distinct endothelium (Text-fig. 4). The amount of undifferentiated mesenchyme present is small, and on the side adjacent to the exocoel the yolk-sac splanchnopleur is lined by a coelomic mesothelium, the nuclei of which, like those of the vascular endothelia, stain intensely in Ehrlich's haematoxylin. A membrane of Reichert is not formed in the yolk-sac wall in the ferret.

THE YOLK-SAC FLUID AND MATERNAL SERUM

Blood samples were collected from some of the hedgehogs and ferrets at autopsy and the concentration of protein nitrogen in the maternal serum was determined. In both the hedgehogs and the ferrets there were differences in the concentrations irrespective of the stage of pregnancy. Differences of a similar order existed in the sera of male hedgehogs which were tested. The protein concentration of the sera of the hedgehogs varied from 6·1 to 7·3 per cent., while that of the ferrets varied but slightly, with a mean value of 5·7 per cent.

(*a*) *the hedgehog*

The presence of a coagulum which stains faintly with aqueous eosin is noticeable in the yolk-sac cavity of hedgehogs in very early stages of pregnancy. In such stages, before the formation of the embryonic knob is completed, the coagulum appears evenly dispersed throughout the yolk-sac cavity. Later it is usually restricted to the periphery of the yolk-sac, being generally more concentrated along the inner margin of the partially inverted yolk-sac splanchnopleur.

TEXT-FIG. 4. A portion of the yolk-sac splanchnopleur of the ferret at 21 days.

Table 1 shows the concentration of protein nitrogen in the maternal sera of one non-pregnant and five pregnant hedgehogs and the concentration in the yolk-sac fluid of four of the pregnant animals.

The volume of fluid obtainable from the yolk-sacs at early stages is too small to be tested. As pregnancy proceeds the volume of the yolk-sac increases, and continues to increase beyond the stage at which the omphaloidean placenta attains its greatest development. Yolk-sac fluid was collected from the uterine swellings of twelve hedgehogs at different stages of pregnancy and tested for the presence of fibrinogen. Two late stages, H23 and H28, gave positive results; all the earlier stages gave negative results. The latest stage to give a negative result was H41 (Table 1), a stage of pregnancy comparable to that illustrated in Text-fig. 1, when the omphaloidean placenta has already passed its greatest development and is retrogressing.

In late stages the yolk-sac cavity is considerably reduced, and it is difficult to dissect out the frozen yolk-sac fluid from the swelling. The fluid obtained from such stages is rather similar in colour to that of the maternal serum. Care was taken to avoid contamination with tissue proteins and maternal blood. At or near term the fluid obtainable is too small to be tested.

At about mid-pregnancy the protein concentration of the yolk-sac fluid is approximately 3 per cent. of that of the maternal serum. This concentration

increases towards the end of gestation; in H23 and H28 it is about 28 per cent. of that of the maternal serum (Table 1).

TABLE 1

The yolk-sac fluid and maternal serum of the hedgehog

Animal number	*Stage of pregnancy*	*Average wt. in gm. of contents of a single yolk-sac*	*Conc. of protein nitrogen (mg./ml.)*		*Reaction with thrombin*
			**Yolk-sac fluid*	**Maternal serum*	
H22	Non-pregnant	..	..	9·85	..
H24	Implanting blastocyst	..	..	10·5	..
H29	Mid-pregnancy	0·181	0·35	11·7	—
H41	Mid-pregnancy	0·195	0·53	10·9	—
H23	Late pregnancy	0·129	2·91	10·5	+
H28	Very late pregnancy	0·074	3·02	10·7	+

* The mean of three determinations in which the variation was not greater than ±1 per cent. of the arithmetic mean. In blank determinations of quantities of nitrogen of the same order as that found in the serum and yolk-sac fluid the variation was of similar degree.

TABLE 2

The yolk-sac fluid and maternal serum of the ferret

Animal number	*Stage of pregnancy*	*Average wt. in gm. of contents of a single yolk-sac*	*Conc. of protein nitrogen (mg./ml.)*		*Reaction with thrombin*
			Yolk-sac fluid	**Maternal serum*	
F10	16½ days	0·189	0·07	8·97	—
F11	17½ days	0·211	0·08	9·18	—
F8	19½ days	0·269	0·09	9·31	—
F9	20½ days	0·250	0·10	9·15	—

* The mean of three determinations in which the variation was not greater than ±1 per cent. of the arithmetic mean. In blank determinations of quantities of nitrogen of the same order as that found in the serum the variation was of similar degree.

(*b*) *the ferret*

The yolk-sac fluid was collected from the uterine swellings of eleven ferrets at stages between the 16th and 21st days of pregnancy. In every case the reaction with thrombin was negative. In a number of animals the fluid contents of the exocoels were also tested, and these too gave negative results. In the ferret the volume of the yolk-sac increases up to about 19 days and subsequently decreases.

The yolk-sac fluid is an extremely dilute protein solution; in some animals a protein precipitate was unobtainable, and on the addition of trichloroacetic acid only a faint opalescence resulted. The concentration of protein nitrogen in the

maternal sera of four of the ferrets was determined, and an attempt was made to estimate its concentration in the yolk-sac fluid of these animals (Table 2). The concentration in the yolk-sac fluid is so low that the results obtained can only be regarded as approximate. The highest protein nitrogen concentration, 0·10 mg./ml., was obtained at 20½ days—about 1 per cent. of the concentration of the maternal serum.

DISCUSSION

In very early stages of pregnancy in *Erinaceus* the decidual tissue adjacent to the blastocyst becomes highly vascular and large blood-spaces are formed in the trophospongia, which is derived from the enlarged cells of the maternal endothelia. Lacunae are formed between the trophoblast and the adjacent decidua, and the maternal blood which circulates in these spaces is thus brought into close proximity with the embryonic tissues, and is separated from the cavity of the yolk-sac only by the bilaminar yolk-sac wall. No such complex organization of vascular channels occurs in the uterine tissue of *Putorius*.

Omphaloidean villi are developed in both forms, but whereas in the ferret they remain avascular until the allantoic mesoderm extends over them, in the hedgehog they are vascularized by vitelline vessels from the area vasculosa. The maternal and foetal circulations are thus brought into close proximity over an increased surface area, and are separated from one another by two cellular layers, the trophoblast and the foetal endothelium. The avascular nature of the omphaloidean placenta of the ferret and its comparative inefficiency as an absorptive organ are probably reflected in the extremely low concentration of protein in the yolk-sac fluid. The highest concentration of protein nitrogen obtained was 0·10 mg./ml. at 20½ days compared with a concentration of 9·15 mg./ml. in the maternal serum. Fibrinogen was absent from the yolk-sac fluid of all the stages of pregnancy that were tested.

In *Erinaceus* the concentration of protein nitrogen in the yolk-sac fluid is considerably higher than that of the ferret. At mid-pregnancy a concentration of 0·35 mg./ml. was obtained, compared with 11·7 mg./ml. in the maternal serum, and fibrinogen was found to be present in the yolk-sac fluid at late stages of pregnancy. In such late stages the volume of the yolk-sac diminishes and the concentration of protein in the yolk-sac fluid increases. The increase in the concentration is probably due, in part, to the absorption of water from the yolk-sac fluid, but since this cannot entirely account for the higher concentrations there must be a continued entry of protein into the yolk-sac during the later stages of pregnancy.

In the rabbit implantation occurs on the 7th day, and by 8½ days a very close relationship has been established between the embryonic tissues and the maternal blood. As soon as the fusion areas are formed maternal blood capillaries grow towards them, and the maternal blood bathes the syncytial trophoblast (Parry, 1950). These fusion areas, which are suggested as the most obvious route

of passage from the maternal blood into the yolk-sac cavity, reach their maximum size and vascularity at $8\frac{1}{2}$ days. During the 7th and 8th days proteins enter the yolk-sac cavity from the maternal blood. It has been demonstrated experimentally that serum albumin enters the yolk-sac at a maximum rate at $7\frac{1}{2}$ days, and that the rate of entry decreases to zero at 9 days. It was suggested that the further entry of serum albumin was prevented by a sudden change in the permeability of the membrane. There is a very rapid build up in the concentration of protein in the yolk-sac fluid between 7 and 9 days. This concentration reaches as high as 40–50 per cent. of that of the maternal plasma, and the fibrinogen which is of maternal origin is present in concentrations between 30 and 40 per cent. of that of the maternal blood (Brambell *et al.*, 1949).

In *Erinaceus* a similar close relationship is established between the maternal blood and the embryonic tissues soon after implantation. At such early stages the yolk-sac cavity is minute, and it is not possible to test its fluid contents. However, at later stages the protein concentration of the yolk-sac fluid is comparatively low and fibrinogen is absent (Table 1). Thus in the rabbit and the hedgehog the early omphaloidean placenta is haemochorial in character, but despite this structural similarity the permeability of the membranes of the two forms must be very different, to account for the high concentration of protein in the yolk-sac fluid of the former, and the relatively low concentration in the yolk-sac fluid of the latter.

Later in development the omphaloidean placenta of the hedgehog is vascularized by the vessels of the area vasculosa, and the foetal and maternal circulations are separated only by the trophoblast and the foetal endothelium. Thus in *Erinaceus* the foetal-maternal vascular relations of the omphaloidean and allantochorionic placentae are similar.

Fibrinogen is present in the yolk-sac fluid in late stages of pregnancy in the hedgehog. In such stages the omphaloidean placenta is rapidly retrogressing, and over its limited extent it is not so highly vascular as in earlier stages. The yolk-sac wall is in contact with the attenuated maternal tissues over the mesometrial pole only, and in this region a thick Reichert's membrane has been formed, separating the endoderm from the trophoblast. It is unlikely, therefore, that entry is effected directly via the omphaloidean placenta since it is in such an advanced state of retrogression. Fibrinogen may enter the yolk-sac by way of the allantochorionic placenta, entering the foetal circulation and thence traversing the vascular endothelia of the vitelline vessels and the endodermal epithelium of the yolk-sac splanchnopleur; or more indirectly by traversing the intervening exocoel and passing through the epithelial layers of the yolk-sac splanchnopleur. Since both these routes are available at earlier stages of pregnancy, it seems unlikely that either is used. It would seem more reasonable to conclude that the fibrinogen is of foetal origin, and that it enters the yolk-sac cavity from the vitelline vessels of the yolk-sac splanchnopleur.

The structure of the yolk-sac splanchnopleur in the later stages of pregnancy

in the hedgehog is very similar to that of the 24-day rabbit embryo (Morris, 1950). In both forms the endodermal epithelium is composed of columnar cells, and the mesenchyme, which becomes progressively more compact, remains undifferentiated except for the vitelline vessels which are formed within it. In the 21-day ferret embryo the cells of the endodermal epithelium are cuboidal and the mesenchyme layer is considerably thinner.

The homogenous membrane that is formed on the inner side of the trophoblast of the mesometrial hemisphere resembles the membrane of Reichert which is found in certain rodents, in the Common and Lesser Shrews (Brambell & Perry, 1945), and in the Indian Musk-Shrew, *Crocidura* (Sansom, 1937). The origin of the membrane in these forms is not clear. Sansom suggests that in *Crocidura* the trophoblast may be responsible for its growth, and that the endoderm of the bilaminar omphalopleur may also contribute to its formation. In *Sorex* Reichert's membrane makes its appearance soon after the formation of the exocoel and before that of the allantois, and it is considered to be of trophoblastic origin (Brambell & Perry, 1945). In *Erinaceus* the membrane appears much later in development, but as in *Sorex* it is first distinguishable in the region of the extra-embryonic mesoderm, between the somatic mesoderm and the trophoblast of the lateral walls of the conceptus. Here it may possibly be formed by the thickening somatic mesodermal cells, or by the trophoblast, or both tissues may take part in its formation. In this region it is completely separated from the yolk-sac endoderm which can have no part in its formation. At the mesometrial pole it becomes noticeable slightly later in pregnancy, between the endoderm and the trophoblast of the bilaminar omphalopleur. These conditions are essentially similar to those which have been described in *Sorex*. Since there appears to be no sufficient reason for supposing that the membrane has a different origin in the different regions of the mesometrial hemisphere of the conceptus, it is reasonable to conclude that in *Erinaceus*, as in *Sorex*, Reichert's membrane is trophoblastic in origin.

The yolk-sac in the ferret and in the hedgehog persists throughout pregnancy, but is considerably reduced in late stages. In *Putorius* the extension of the exocoel completely separates the yolk-sac splanchnopleur from the chorion, so that omphaloidean placentation is terminated at about 21 days. In *Erinaceus* the mesoderm does not complete its extension mesometrially, and although the omphaloidean placenta persists to term, the exocoel separates the vascular splanchnopleur from the somatopleur, so that the yolk-sac placenta in the very late stages is bilaminar and avascular.

SUMMARY

1. In *Erinaceus*, the hedgehog, a close relationship between the maternal blood and the embryonic tissues is established soon after implantation by the organization of vascular channels between the trophoblast and adjacent decidua.

No comparable organization of vascular channels occurs in the uterine mucosa of *Putorius*, the ferret.

2. In *Putorius* the omphaloidean placenta is avascular, and its probable inefficiency as an absorptive organ is reflected in the extremely low concentration of protein in the yolk-sac fluid. Fibrinogen was absent from the yolk-sac fluid in all the stages of pregnancy that were tested.

3. In *Erinaceus* the omphaloidean placenta, like the allantochorionic placenta, is haemochorial in character. At its optimum development it is highly vascular. The concentration of protein in the yolk-sac fluid of the hedgehog is considerably higher than that in the ferret, but fibrinogen is absent except in very late stages of pregnancy, when the omphaloidean placenta is in an advanced stage of retrogression. It is probable that in these late stages the fibrinogen is of foetal origin.

4. Despite the structural similarity which exists between the early omphaloidean placenta of the hedgehog and that of the rabbit, which is also haemochorial, there is no comparable rapid build up in the concentration of protein in the yolk-sac fluid of the former such as occurs in that of the latter. The permeability of the membranes must be very different in the two forms.

5. Reichert's membrane is formed late in development in the hedgehog, and it increases in thickness in very late stages of pregnancy. It appears to be of trophoblastic origin. Reichert's membrane is absent in the ferret.

The main part of this work was carried out at the Department of Zoology, University College of North Wales, during the tenure of an Agricultural Research Council Studentship, for which I wish to express my thanks. The author is much indebted to Professor F. W. R. Brambell, F.R.S., for his advice and encouragement, and also to Mr. W. A. Hemmings.

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EXPLANATION OF PLATE

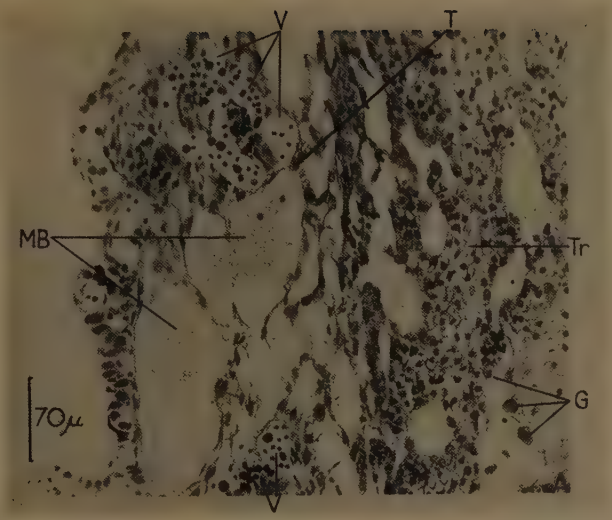
FIG. A. A portion of the vascular yolk-sac placenta of the hedgehog at its greatest development. Vitelline vessels have penetrated into the spongy trophoblast almost as far as the trophospongia.

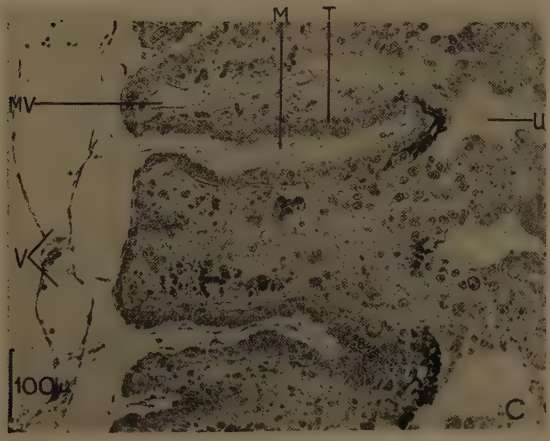
FIG. B. A portion of the lateral wall of the conceptus at a late stage of pregnancy, showing the membrane of Reichert between the thickened cells of the somatic mesoderm and the trophoblast and trophospongia.

FIG. C. A portion of the yolk-sac placenta of the ferret at 17 days. The trophoblastic villi have become hollowed out and are partially filled with mesenchyme cells. Accumulations of degenerating maternal tissue, associated with the tips of the villi, are being displaced into the lumina of the uterine glands.

Abbreviations used in the plate:

G. Giant nucleus.
M. Mesenchyme.
MB. Maternal blood.
MV. Maternal vessel.
R. Reichert's membrane.
S. Somatic mesoderm.
T. Trophoblast.
Tr. Trophospongia.
U. Uterine gland.
V. Vitelline vessel.





B. MORRIS
Plate 1

Changes in Deoxyribonucleic Acid and Total Nitrogen in Planarian Worms during Starvation

by E. HOFF-JØRGENSEN, EBBA LØVTRUP, *and* S. LØVTRUP

From the Institute of Biochemistry, University of Copenhagen, and Cytochemical Department, Carlsberg Laboratorium, Copenhagen

INTRODUCTION

WHEN planarian worms are subject to starvation their volume decreases, and the fact that they can survive even when their volume has been reduced to a small fraction of the original, sometimes to less than 1 per cent., has made these animals the object of several investigations of starvation effects. One question to which special attention has been paid is whether, in the starved animals, it is the number or the size of the individual cells, or both, that have been reduced in correspondence with the reduced volume. The previous results pertaining to this question will be dealt with in the discussion, where it will be shown that quite different answers have been obtained.

Recent biochemical investigations of the effects of starvation on various organs and tissues in mammals (cf. the review by Davidson & Leslie, 1950) have demonstrated that the content of deoxyribonucleic acid (DNA) remains completely constant under conditions of starvation, whereas considerable losses occur in weight and other quantities. These results indicate, despite some ambiguity in interpreting the meaning of constancy of DNA, that the main effect of starvation is a reduction of the volume of the individual cells rather than a decrease of their number.

Since these results have some bearing on the problem stated above, it was decided to follow the changes in total nitrogen (TN) and in DNA in starving planarian worms.

MATERIAL AND METHODS

Individuals of the freshwater species *Polycelis nigra*, collected in a lake at the beginning of October, constituted the experimental material. The animals were kept in the laboratory in flat dishes in tap-water at room temperature (about 20° C.). The water was changed frequently, and at the same time the dishes were carefully cleaned.

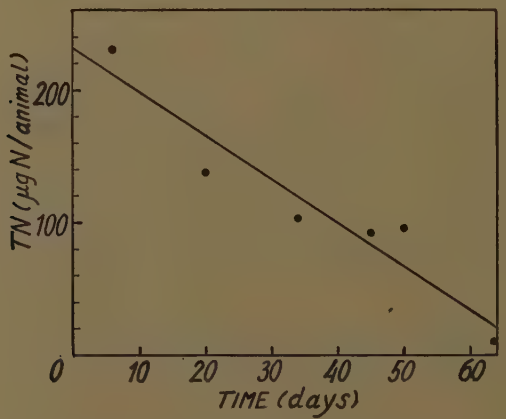
[J. Embryol. exp. Morph. Vol. I, Part 2, pp. 161–5, June 1953]

To make each determination of nitrogen content twenty-five animals were digested in a mixture of 1 ml. H_2SO_4, 0·7 g. K_2SO_4, and 0·07 g. $CuSO_4$. After digestion was completed, the digests were diluted to about 100 ml. and the content of ammonia determined by Nesslerization.

For DNA determinations twenty-five individuals were dried with acetone. The dry powder was incubated with 1·0 ml. 0·5 N. NaOH at 100° C. for 15 minutes. Afterwards 5 ml. of a buffer-activator solution (0·06 M. maleic acid; 0·01 M. $MgSO_4$) was added. After addition of 100 μg. crystalline deoxyribonuclease in 0·1 ml. water, the mixture (pH 6·8) was incubated at 37° C. for 24 hours. The mixture was then diluted to 100 ml., centrifuged, and the amount of deoxyribonucleosides determined microbiologically (Hoff-Jørgensen, 1951). The basis of this method is that *Thermobacterium acidophilus* R26 can only grow in the presence of deoxyribosides. Under standardized conditions the extent of growth will be a function of the deoxyribosides added to the medium, and the amount of these latter can thus be determined by measuring turbidimetrically the bacterial growth.

RESULTS

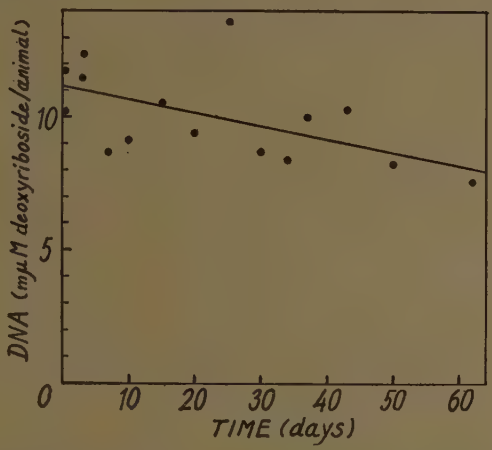
The experiments lasted for 64 days, during which time the average length of the animals (stretched) decreased from about 15 mm. to about 5 mm. The results



TEXT-FIG. 1. Changes in total nitrogen during starvation of *Polycelis nigra*. Each point is the mean of 25 animals. The regression has the following equation: $y = 112 - 3{\cdot}31(x - 36{\cdot}3)$.

of the nitrogen determinations have been plotted in Text-fig. 1. The line drawn in the figure is the regression calculated by the method of least squares. The value of the regression coefficient is $3{\cdot}31 \pm 0{\cdot}54$. The regression is highly significant ($P < 0{\cdot}01$), and indicates that only about 14 per cent. of the original amount of TN is left after 60 days of starvation.

The results of the DNA determinations, together with the regression line calculated from them, are shown in Text-fig. 2. The value of the regression coefficient is $0{\cdot}047 \pm 0{\cdot}021$. The slope of the regression line hardly differs significantly from zero ($0{\cdot}05 < P < 0{\cdot}10$), and there is therefore no reliable evidence that DNA changes during starvation.



TEXT-FIG. 2. Changes in DNA (deoxyribosides) during starvation of *Polycelis nigra*. Each point is the mean of 25 animals. The regression has the following equation: $y = 10{\cdot}1 - 0{\cdot}047(x - 22{\cdot}6)$. 1 mμM. deoxyriboside corresponds to about 0·327 μg. DNA.

The scatter of the individual determinations is far beyond the experimental error, and demonstrates very great individual variations in the content of DNA per animal. It is possible that less varying results could have been obtained if some correction for the size of each sample had been used, e.g. dry weight.

If the amount of DNA is proportional to the number of nuclei, our results indicate that the average content of TN per nucleus (and probably per cell) decreased during 2 months of starvation to approximately 20 per cent. of the initial value.

DISCUSSION

The authors who have studied the morphological effects of starvation in planarian worms disagree about the fate of the individual cells. Schultz (1904) on the basis of his studies on *Planaria lactea* advanced 'the law, valid for planarian worms', that only the cell number, and not the cell size, was reduced when the total volume was reduced as a result of starvation. In starved animals Schultz observed various types of degenerating cells, from reduction in volume to necrosis, and these were found in all organs and tissues investigated. He found, in agreement with later observations, that the sexual organs were relatively more

reduced than other organs or tissues. Stoppenbrink (1905) found that the decrease in cell size almost corresponded to the decrease in total volume (*Planaria alpina, Dendrocoelum lacteum, Polycelis nigra,* and *Planaria gonocephala*). Only in the sexual organs did Stoppenbrink observe necrosis of cells to any measurable extent. Child (1915) found that both reduction of cell size and cell number occurs (*Planaria dorotocephala*). The same was found by Abeloos & Lechamp (1929), who measured the length of epithelial cells of *Planaria gonocephala* of different size, during growth and during reduction due to starvation. Since they did not find proportionality between total length and cell length, they concluded that both cell size and cell number changed.

The remarkable stability of DNA under conditions of starvation, which has been found in other objects by chemical analyses, is partly supported by the present results, even though they do not exclude a small decrease. It is desirable in future work to get this point definitively settled, because the previous chemical investigations have been made on single organs and tissues, while in the present instance whole animals have been used. The morphological investigations by Schultz and by Stoppenbrink mentioned above emphasize the possibility that in whole animals some organs may degenerate completely, thus releasing their DNA-content, while other organs or tissues, despite being reduced in volume, may preserve their original number of cells and their DNA. In this possibility may lie the clue to the apparent difference between our results and the chemical investigations just mentioned.

In this connexion it should be stressed that the method used by us is specific for deoxyribosides, i.e. not only the highly polymerized DNA, but all kinds of breakdown products would be registered as 'DNA'. Before a degeneration of a cell nucleus, including depolymerization of the DNA, is to be registered as loss in 'DNA' by our method, the breakdown products must either be lost from the animals, e.g. by diffusion, or the deoxyribosidic linkages must be broken.

It is difficult to make a comparison between our results and the morphological results mentioned above, since these contain no quantitative data. It might also be argued that such a comparison in any case is excluded because different species were used. Against this argument it may be said that Stoppenbrink, who worked with several species, does not mention any species differences, and also that the phenomenon investigated is so fundamental that it would seem rather improbable that such species differences occur.

Our results do not rule out any of the results published on the basis of morphological studies, but they make the conclusion at which Schultz arrived, i.e. that the reduction in volume is due to reduction in overall cell number, seem rather dubious.

Since our results do not exclude a small loss of DNA, they are compatible with Stoppenbrink's finding of a degeneration confined to the sexual organs. Our results would also agree with those of Child (1915) and of Abeloos & Lechamp (1929).

SUMMARY

1. The changes in total nitrogen and in deoxyribosides (DNA) have been followed during starvation of the planarian worm, *Polycelis nigra*.

2. It was found that total nitrogen content after 60 days had been reduced to about 14 per cent. of its initial value.

3. It is not possible to exclude with certainty the occurrence of any DNA loss, but it is clear that the loss, if any, is much smaller than the loss in nitrogen.

4. The implications of these results for previous morphological investigations of the effects of starvation on planarian worms are discussed.

We are happy to acknowledge our indebtedness to Professor H. V. Brøndsted, whose work on planarian worms has called our attention to this interesting field of experimental biology.

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A Note on Abnormalities induced in Mouse Embryos by Trypan Blue

by C. H. WADDINGTON *and* T. C. CARTER[1]

From the Institute of Animal Genetics, Edinburgh

INTRODUCTION

In 1948 Gillman, Gilbert, Gillman & Spence reported the results of experiments in which rats which had been injected at fortnightly intervals with 1 c.c. of 1 per cent. trypan blue were allowed to produce offspring. A most remarkable variety of anatomical abnormalities, often of a very severe character, was found in the young. The malformations included hydrocephalus, spina bifida, eye and tail defects, and alterations in internal organs. Some of the defects were similar to abnormalities which can be produced by mutant genes, either in the rat or in other mammals, and could therefore be regarded as belonging to the ill-defined class of 'phenocopies'. Since the mammal embryo is notoriously difficult of access, and comparatively little is known of its developmental physiology, the phenomena discovered by the South African authors seemed worthy of further investigation. Moreover, there is a considerable intrinsic interest in the possibility of the induction of pathological development by influences emanating from the maternal body, particularly in connexion with the fact that certain virus infections of the mother may be followed by effects of this kind (Gregg, quoted by Gillman, Gilbert, Gillman & Spence, 1948). We therefore decided to make an exploratory study of the effects of trypan blue in the mouse embryo. Since this did not reveal such striking modifications of development as were found in the rat, it has not seemed worth while to carry the investigations into all the possible ramifications of time and dosage, and the results obtained so far are here put on record. A preliminary note on the work has appeared elsewhere (Waddington & Carter, 1952).

MATERIAL AND METHODS

Since some of the abnormalities which may be produced by treatment with trypan blue may be expected to simulate genetic effects, it is desirable to carry out the experiments with strains of animals in which there is some reason to believe that mutant genes of the relevant kinds are absent. For this reason, and also because they were more easily available, we have worked with mice instead

[1] Member of the scientific staff of the Medical Research Council. *Authors' address*: Institute of Animal Genetics, West Mains Road, Edinburgh 9, Scotland.

[J. Embryol. exp. Morph. Vol. I, Part 2, pp. 167–80, June 1953]

of the rats used by Gillman *et al.* The strain employed was the *CBA* inbred strain, in which gross abnormalities (such as hydrocephalus, tail abnormalities, &c.) do not occur with any appreciable frequency.

Gillman and co-workers injected their rats at fortnightly intervals, the times of injection not being related in any specific way to the time of conception of the offspring. In the analysis of their results they found that the greatest effect was produced in those animals which happened to have been injected on the seventh or eighth days of pregnancy; and the effect was greater still if there had been an earlier injection a fortnight previously. We decided to concentrate attention on the effects of injections given at the time of maximum efficiency. After some preliminary trials the following procedure was adopted. Females were left with males overnight, and examined next morning for copulation plugs. Seven days after the plug was found a single subcutaneous injection of 0·5 c.c. of a 1 per cent. aqueous solution of purified trypan blue was given.

In the first series of experiments four of the injected females were killed each day and the embryos dissected out, starting one day after the injection; that is to say, embryos were obtained at daily intervals between $8\frac{1}{2}$ and $16\frac{1}{2}$ days after conception. If a female contained no implanted embryos it was discounted and a new one prepared in place of it. A similar number of non-injected females were dissected in the same way to serve as controls. Embryos were fixed in Bouin's fluid, stained in Delafield's haematoxylin or borax carmine, and cleared and examined in methyl benzoate. A certain number of representative specimens were sectioned for more detailed study.

In a second series of experiments the same procedures were followed, but the females were allowed to come to term and to bear their litters, which were examined as soon after birth as possible.

RESULTS

A. *First Series*

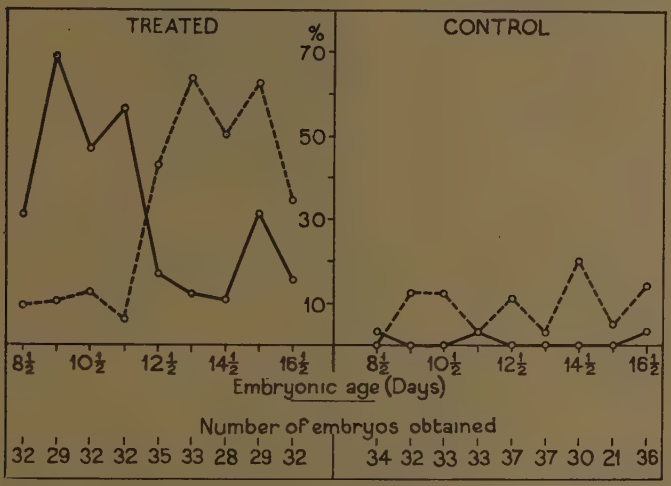
The general results of the experiments are shown in Text-fig. 1. It will be seen that, as was to be expected, the controls showed comparatively few abnormal or dead embryos, the number of prenatal deaths being well within the normal range for the mouse. The only marked anatomical abnormality was in one embryo which was unilaterally microphthalmic.

In the treated series, on the other hand, a very considerable number of abnormal or dead embryos were found. Already in the $8\frac{1}{2}$-day embryos (1 day after injection) the percentage of abnormals was considerably higher than in the controls. In the next few days the percentage of abnormals rose considerably, to fall again sharply in the $12\frac{1}{2}$-day and later samples, by which time the frequency of dead embryos had greatly increased. Clearly many of the embryos which show abnormalities in the earlier period die before the end of pregnancy.

The abnormal embryos exhibited a considerable variety of conditions. It will perhaps be best to describe the appearances characteristic of each successive day

of development before attempting to arrange the abnormalities into connected causal sequences.

8½-day embryos. In the normal 8½-day embryo the main axis of the body has only recently appeared, and comparatively few somites are formed, so that there is not yet much scope for morphological irregularities. In the treated embryos there is often a suggestion that development has been somewhat retarded, but the normal variation is fairly wide and it is difficult to be certain that the inhibited embryos are outside the normal range. The most striking phenomenon in the



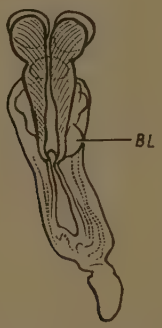
TEXT-FIG. 1. Numbers of embryos, including dead implantation sites, obtained at each stage in the experimental (left) and control series; and percentages of the total which were abnormal (full line) or dead (broken line).

embryos dissected out of the uterus is the concentration of blue dye in the central region of the egg cylinder, the future yolk-sac, which may be very deeply stained. Both the embryo proper and the amnion remain of their normal colour and do not take up the dye to any noticeable extent.

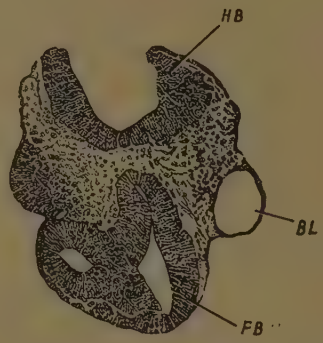
The dead embryos which are found at this stage are usually already disintegrated and appear as small more or less featureless lumps of necrotic tissue. They probably represent the amount of mortality which is normal in this strain.

9½-day embryos. The blue staining of the yolk-sac is still very noticeable. It is also quite clear that in many embryos the general rate of development has been slowed down, the stage of closure of the neural folds and the number of somites formed being definitely less than would be expected in a normal 9½-day embryo. Morphological abnormalities are also obvious in many cases. The neural tube often closes abnormally, remaining open in the brain region at a time when its condition elsewhere would lead one to expect it to be closed. It may also not be

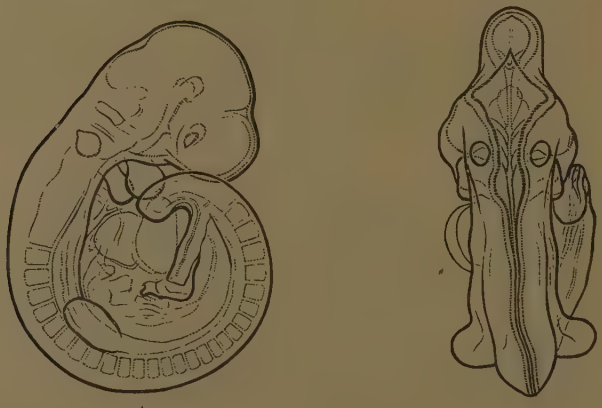
stretched out straight, but show kinks and bends. Sub-epidermal blisters, normally fairly small, appear in some embryos, particularly on the sides of the hind-brain or in the thoracic region, above or lateral to the first few pairs of somites (Text-figs. 2 and 3).



TEXT-FIG. 2. 9½-day unturned embryo, experimental series, seven-somite stage, dissected out of its amnion; it shows a subectodermal bleb (BL) on the right side in the somite region.



TEXT-FIG. 3. 9½-day turned embryo, experimental series, sectioned almost transversely in the head, showing subectodermal bleb (BL) between the fore-brain (FB) and hind-brain (HB).

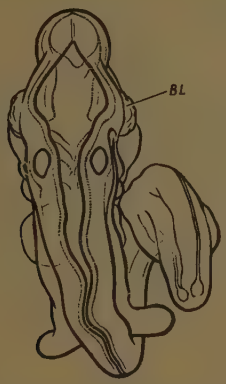


TEXT-FIG. 4. 10½-day normal embryo, control series. *A*, seen from the right side; *B*, the dorsal aspect of the myelencephalon.

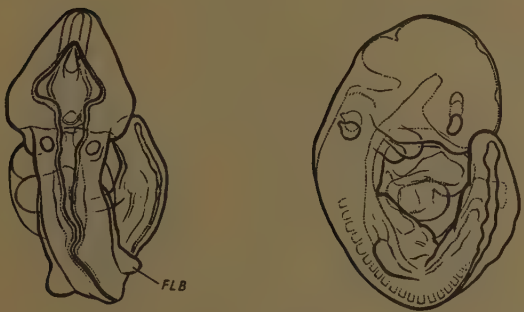
In the normal 9½-day *CBA* embryo the turning process occurs, whereby the embryo comes to be rolled up into a complete gyre of a right-handed spiral, so that the dorsum of the lumbar region faces in the opposite direction to that of the myelencephalon. The result of this rolling up is shown in Text-fig. 4. In many

abnormal embryos the posterior part of the body, from about fore-limb level, is deflected to the right; the dorsum of the lumbar region then faces to the right of the embryo, or even in the same direction as that of the myelencephalon (Text-figs. 5 to 8).

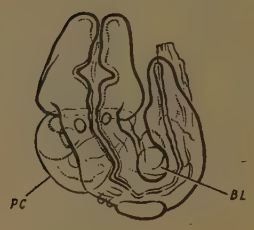
10½- and 11½-day embryos. The blue staining of the yolk-sac is still visible, but is becoming less intense except in the region near the placenta. There are still some very strongly retarded embryos in which the neural folds are not yet completely closed. Sub-epidermal blebs may also still be found.



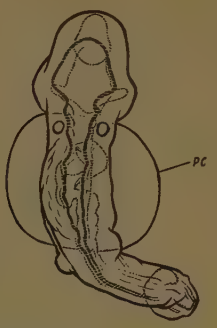
TEXT-FIG. 5. 10½-day embryo, experimental series, showing bleb (BL) on each side of the head near the maxillary process, dilated mid- and hind-brain, and slight convolution of the neural tube at the level of the heart.



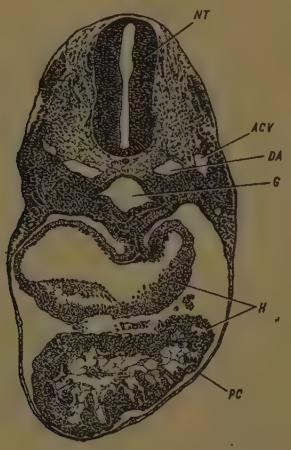
TEXT-FIG. 6. 10½-day embryo, experimental series, showing enlarged pericardium, body posterior to the fore-limb buds (FLB) deflected to the right, head oedematous, hind-brain dilated, and neural tube somewhat convoluted.



TEXT-FIG. 7. 10½-day embryo, experimental series, showing retarded development, enlarged pericardium (PC), subectodermal bleb (BL) dorsal to right fore-limb bud, failure of turning, oedematous head, failure of neural folds to close in the head, and convolution of the neural tube at the level of the heart.

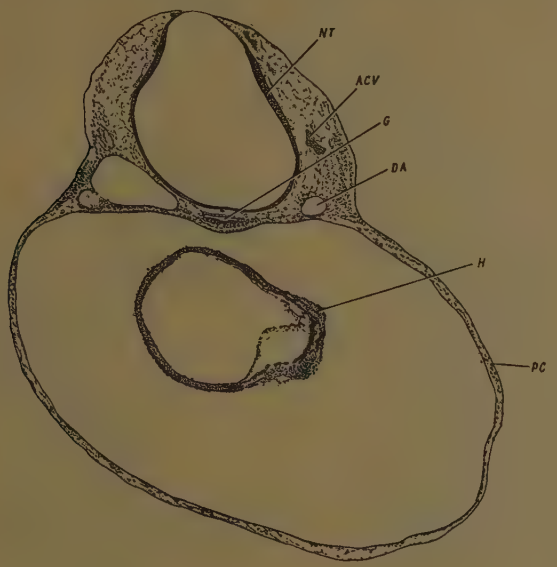


TEXT-FIG. 8. 11½-day embryo, experimental series, showing retarded development, greatly enlarged pericardium (PC), posterior part of the body deflected to the right, head oedematous, mid- and hind-brain dilated, and neural tube convoluted.



TEXT-FIG. 9. 10½-day embryo, control series, sectioned transversely at the level of the heart. For comparison with Text-fig. 10. ACV, anterior cardinal vein; DA, dorsal aorta; G, foregut; H, heart; NT, neural tube; PC, pericardium. The rest of the section, which shows also the hind-limb region and tail-bud, has not been drawn.

The most characteristic features of the abnormal embryos of this age, however, are four effects which it seems probable are closely connected with one another. These are (i) an enormous dilation of the pericardium, sometimes but not always accompanied by a corresponding increase in the size of the heart; (ii) a swelling of the embryonic blood-vessels, and general oedema of the tissues, affecting particularly the head; (iii) a deflexion of the posterior part of the body



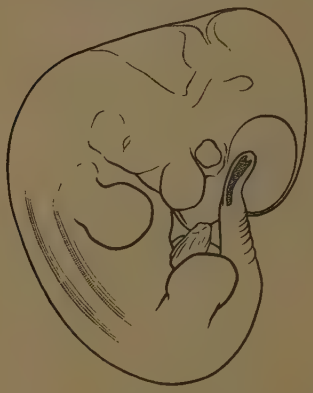
TEXT-FIG. 10. 11½-day embryo, experimental series, sectioned as in Text-fig. 9. Note the enormous pericardium, heart cut only once, dilated left dorsal aorta, small gut, and general sparseness of the tissues, especially the dilated neural tube. Abbreviations as in Text-fig. 9.

to the right side; and (iv) dilation of the neural tube, which is often kinked. Illustrations of these effects are shown in Text-figs. 5 to 8, from which their character will be sufficiently obvious without further description in words. The enlarged pericardium may reach extraordinary dimensions, so that the embryo looks almost like that of a teleost, with the pericardium taking the place of the teleost yolk-sac. This may be seen in Text-figs. 9 and 10, in which an abnormal and retarded embryo is compared with a normal one of about the same stage of development.

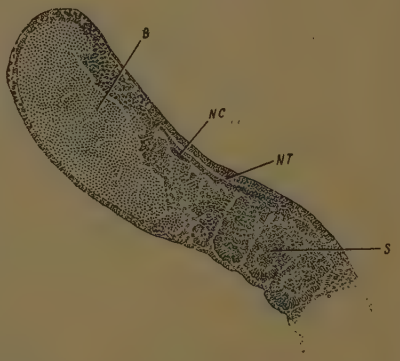
12½-day embryos. In the later 11½-day embryos, in which the posterior part of the body has been deflected to the side, it is usually small and retarded in development. By 12½ days this region has become very abnormal and is usually

necrotic. In these cases death of the whole embryo seems to occur towards the end of this period.

In those embryos in which the turning is more satisfactorily completed, morphological abnormalities are usually comparatively slight. They are more or less confined to the appearance of haematomata, particularly on the tail and in the head, although the tail may also show phenomena suggestive of degeneration without any marked escape of blood into the tissues. An example is shown in Text-fig. 11; Text-fig. 12 illustrates a section through a tail haematoma; Text-fig. 13 shows a section through a tail in which the tail-gut is deflected.



TEXT-FIG. 11. 12½-day embryo, experimental series, showing extravasation in the tip of the tail.



TEXT-FIG. 12. 12½-day embryo, experimental series; longitudinal section through the tip of the tail, showing large conglomeration of blood (B). NC, notochord; NT, neural tube; S, somite.

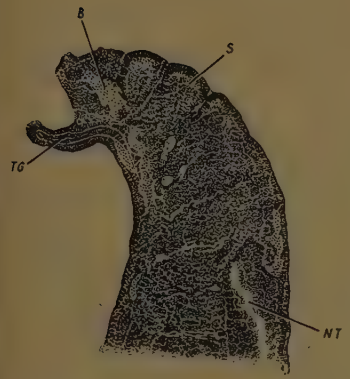
Later embryos. In embryos which survive till after the thirteenth day, the only abnormalities which have been found are shortening and other defects of the tail, with or without the extravasation of blood, and the occasional occurrence of haematomata on other sites. Among 58 embryos which survived till 13½ days or more, 17 had shortened tails, with or without haematomata on the head in addition, and 3 had head haematomata with normal tails. Sections of shortened tails show regions of necrosis near the tip and sometimes abnormal relationships of the tail-gut or notochord (Text-figs. 13 and 14).

The only other abnormality was a single case of otocephaly.

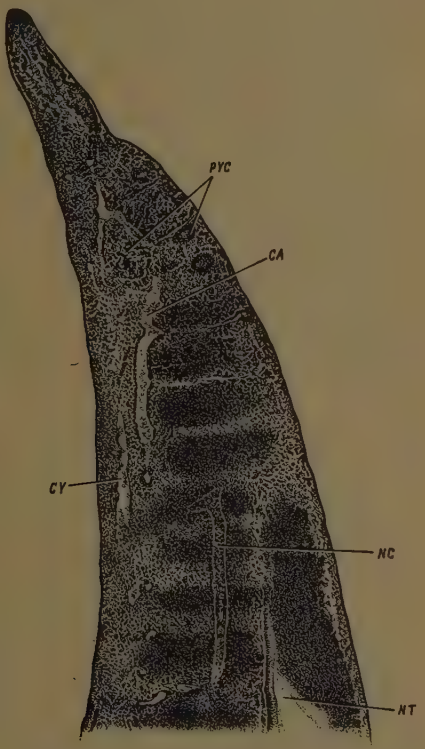
B. *Second Series*

The litters produced by those animals which were allowed to bring their young to term were usually small; and many of the new-born mice were eaten by their mothers in whole or in part. The numbers of animals examined are shown in the Table. The abnormals among the treated litters were mostly animals with tail

defects (shortening or bending). Some also exhibited head haematomata. A few developed corneal opacity, but since this condition has been seen in untreated related stocks, the occurrence of the defect here cannot be certainly attributed



TEXT-FIG. 13. 12½-day embryo, experimental series; longitudinal section through the tip of the tail, showing conglomeration of blood (B) and, immediately proximal to it, ventral branch of the tail containing tail-gut (TG). NT, neural tube; S, somite.



TEXT-FIG. 14. 13½-day embryo, experimental series; longitudinal section through the tail, showing deflected notochord (NC) and pycnotic tissue (PYC) near the tip. CA, caudal artery; CV, caudal vein; NT, neural tube.

to the trypan blue injections. The three abnormals among the controls consisted of two cases of head haematomata and one with club foot.

The data recorded in the Table show a very marked deficiency of females among both the normals and abnormals in the treated litters. Whereas of the classifiable young in the control litters there were 86 ♀ to 102 ♂, in the treated litters there were only 15 ♀ to 49 ♂. It is most plausible to attribute this disproportion to a greater relative frequency of death and resorption of female embryos.

DISCUSSION

The malformations of the embryos in the first series exhibit a rather considerable variety, and it is of interest to consider whether all the different abnormalities can be regarded as consequences of one original effect of the dye. The fact that almost immediately after the injection the dye can be seen to be present in high concentration in the yolk-sac gives us some clue as to the kind

TABLE

Litters carried to term

(*A* = abnormal, *N* = normal, ? = partly cannibalized)

Series	*Females with plugs*	*Litters of zero*	*Progeny examined* ♀ *N*	♀ *A*	♀ ?	♂ *N*	♂ *A*	♂ ?	?	*Total*	*Mean litter size*
Treated .	31	8	10	4	1	26	13	10	7	71	2·29
Control .	43	9	84	1	1	99	2	1	7	195	4·53

of basic effect which it would be most reasonable to invoke. The yolk-sac comes into contact with Reichert's membrane, and through it the maternal decidua, at an early stage; and in rodent embryos it probably then forms the main channel of nutrition. An interference with its function might well be expected to lead to disturbance of the nutrition of the embryo, and this may account for the general inhibition of growth and differentiation which is a marked feature of many of the abnormal embryos of $9\frac{1}{2}$ and $10\frac{1}{2}$ days. Another major category of abnormalities can, very broadly, be described as affecting the body fluids or circulatory system of the embryo. In this type we may include the formation, in early stages, of sub-epidermal blisters or blebs filled with clear fluid and located usually on the head or trunk, and the subsequent dilation of the neural tube. The haematomata which appear in rather later embryos are also local accumulations of body fluid, in this case of blood. They, like the earlier blisters, are formed more or less superficially, for instance under the epidermis of the head. When they form, as they often do, in the tail, they are in a small, rapidly growing region of the body, and it is only to be expected that for merely mechanical reasons their presence would cause considerable disturbance of the organization of the tail, e.g. in the direction of growth of the notochord or tail-gut, which might easily account for the kinks, bends, and shortening seen in the newborn.[1]

[1] Experiments on the effect of trypan blue on amphibian embryos, conducted while this paper was in the press, have shown that in those forms the dye suppresses the differentiation of neural structures and of the notochord. One must therefore keep in mind the possibility that the comparatively slight abnormalities of those organs seen in the mouse embryos may not be only secondary consequences of mechanical disturbance but may in part be produced by a direct action of the dye.

An even more drastic effect on the embryonic body-fluid system is revealed by the enlargement of the pericardium, heart, and embryonic vessels particularly in the head region. The oedema of the head is presumably also a part of this syndrome. It is the enlargement of the pericardium which is carried to the most extravagant degree. This structure is often hypertrophied out of all proportion to the heart, even when the latter is somewhat enlarged.

There would seem to be two alternative ways of regarding the whole complex of effects on the embryonic body fluids. On the one hand, one might suppose that the primary effect was on the nature or quantity of the fluids (for instance by a decreasing of the passage of maternal substances into them through the yolk-sac) and that the formations of blisters, the hypertrophy of the heart, the swelling of the blood-vessels, and the oedema were secondary consequences; or one might suppose that the primary effect was a direct morphological alteration in the heart region, leading to hypertrophy of the heart itself and of the pericardium, and that the circulatory disturbances were secondary consequences of this. In many ways the former alternative would seem the more attractive, although the enormous expansion of the pericardium is not quite easy to fit into such a scheme. But the matter can hardly be settled by the kind of evidence yielded by these experiments.

The enlargement of the pericardium, whether as a primary or secondary effect, would seem to provide the explanation for most of the other abnormalities which remain to be discussed. It is clear that the failure of the normal turning movement is closely connected with the presence of the enlarged organ in the very region towards which the tail bud usually moves. The occurrence of thoracic blisters may also sometimes impede the movement. It is to the failure of the turning, and to the consequent abnormal bending of the axis and impeding of the circulation to which this leads, that one can attribute the later degeneration of the posterior end of the body and the eventual death of many embryos at about $12\frac{1}{2}$ days.

The only types of abnormality which have not so far been fitted in to this scheme are the delay of closure of the neural folds in the head region and the appearance of kinks or convolutions in the neural tube. The first of these is most probably a relatively unspecific effect of general inhibition. A similar failure of closure is of common occurrence in chick embryos grown under abnormal conditions, as for instance *in vitro* (Waddington, unpublished observations). Kinks of the neural tube are also often seen in such embryos and are probably to be attributed to disproportionate growth, such as may result when a general inhibition affects the surrounding tissues more severely than it does the neural tube itself. Thus both the neural effects can probably be considered as consequences of general retardation, and do not call for the postulation of a specific and particular cause.

It therefore appears probable that the whole range of abnormalities can be accounted for in terms of a general inhibition of development acting at about the

time that the circulation appears and a more specific effect on the body fluids and the associated circulatory organs.

It is to be noted that these effects begin to come into operation very soon after the dye is injected. We have seen that the second series of experiments gives evidence of a greater sensitivity in female than in male embryos; it is presumably operative before $12\frac{1}{2}$ days, at which time the greatest uterine mortality takes place. In fact it would seem likely that the difference in sensitivity is exhibited in the $8\frac{1}{2}$-day and $9\frac{1}{2}$-day embryos in which the abnormalities are initiated which may eventually lead to death *in utero*. At such a time the sex differentiation of the embryos has not yet made its appearance, and the greater sensitivity of females than of males must be one of the earliest sex differences yet discovered.

Simultaneously with the work reported in this paper, a rather similar investigation has been carried out by Hamburgh and has been briefly reported (1952). He used an inbred Bagg Albino strain of mice, and gave two injections, each of $\frac{1}{4}$ to $\frac{1}{2}$ c.c. of 0·5 per cent. trypan blue, the first some 8 days before fertilization and the second 7 to 8 days after it. He also found that the main abnormalities in the young born after such treatment affected the tail, and that in the 10- to 14-day embryos abnormalities of the anterior part of the axis also occurred. He appears, in fact, to have encountered cases of pseudencephaly, which denote the survival, to a much later stage than in our experiments, of embryos with a profound disturbance of the anterior part of the neural system. He does not, in the preliminary communication, draw attention to any hypertrophy of the pericardium, or of the heart and embryonic blood-vessels, and he does not mention any failure in the turning of the posterior end of the body. The publication of a full account of his experiments will be awaited with interest; it may prove that his stock of mice reacts differently to ours, either because of genetic differences, or as a consequence of the difference in the timing and concentration of the injections.

The effects reported here, as well as those of Hamburgh, differ from those of Gillman, Gilbert, Gillman & Spence in the fact that the doses used on mice have had more profound effects and led to the death *in utero* of all the more extreme abnormalities, so that only minor tail defects remain alive till birth. It is difficult to tell whether other dosages, which allowed more abnormal embryos to struggle through to term, would have produced embryos showing the same types of defect as those authors described in their rats. It may perhaps be doubted whether this would have been the case, at least with the *CBA* mice. Gillman *et al.* found many cases of pseudencephaly and spina bifida, whereas the data from our series suggest that the disturbance of the turning movement would always prove lethal before the neural tube was affected as drastically as that. It may be that this is a result of the particular time at which our injections were given, but it must be remembered that the time of closure of the neural folds and the time of embryonic turning are not very different, and it is perhaps more plausible to suggest that there is a real difference in the pattern of sensitivity between rats and *CBA* mice. The fact that Hamburgh observed pseudencephaly among his

embryos may indicate that Bagg Albino mice are more similar to rats in this respect.

Ancel (1947) has described experiments in which trypan blue was injected into chick eggs. A certain similarity with our results is shown in the fact that the dye had a primary effect on the circulation, causing haemorrhages, which in the chick embryos often led to the local destruction of the body wall and the appearances of hernias of the type known as coelosomy.

SUMMARY

1. Females of an inbred *CBA* strain of mice, kept in the presence of males, were injected with $\frac{1}{2}$ c.c. of 1 per cent. trypan blue solution 7 days after the detection of a copulation plug. In one series of experiments the animals were killed and the embryos recovered 1, 2, 3, &c., days after the injection; in another series the females were allowed to bring their embryos to term.

2. The injected dye soon becomes accumulated in the yolk-sac, and a little later is visible in the region of the placenta.

3. In many $9\frac{1}{2}$-day embryos the rate of development has been slowed down; the closure of the neural tube is abnormal, and the tube itself may show bends and kinks. Sub-epidermal blebs may be found in various regions.

4. The rolling up of the embryo into a right-handed spiral, which normally occurs at about $9\frac{1}{2}$ days, is very often affected, the posterior part of the body being deflected to the right. At the same time, the pericardium begins to be very markedly inflated; and it seems probable that the abnormal turning of the embryo is caused by the mechanical impediment presented by the hypertrophied pericardium.

5. There is often a considerable dilation of the embryonic blood-vessels, particularly in the head; and fluids tend to escape from the vessels. There are often haematomata in the tail region.

6. There is a high death-rate of embryos at 12–13 days. Those which survive to term usually show few abnormalities other than shortening or kinking of the tail.

7. There is a marked shortage of females in the surviving young. This is presumably due to a differential death-rate, and since many deaths occur by 12 days, the females must be more sensitive than the males at a still earlier period, well before the time at which sexual differentiation takes place.

8. It is suggested that the main effects of the dye are firstly a general inhibition of development and secondly a more specific effect on the body fluids and circulatory organs.

We should like to express our gratitude to Miss M. M. Manson and Miss E. Paton for technical assistance; to Mr. E. D. Roberts, who drew the figures; to Messrs. Imperial Chemical Industries (Dyestuffs Division) Ltd. for a sample of

purified trypan blue, and to the Agricultural Research Council for financial support of the general work of the laboratory.

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Journal of
Embryology and Experimental Morphology
[J. Embryol. exp. Morph.]

VOLUME 1 *June 1953* PART 2

Contents

PRINTED IN GREAT BRITAIN AT THE UNIVERSITY PRESS, OXFORD
BY CHARLES BATEY, PRINTER TO THE UNIVERSITY